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(54) Title: *IN VITRO* AND *IN VIVO* GROWTH-PROMOTING PROTEINS AND PEPTIDES FOR KIDNEY EPITHELIAL CELLS

(57) Abstract

Novel growth peptides derived from protein factors having molecular weights of about 22 and 45 kDa stimulate mitogenic activity of epithelial, but not fibroblastic cells, in particular, kidney epithelial cells, both *in vitro* and *in vivo*. A source of the factors is scrape-wounded kidney epithelial cells in culture. Synthetic peptides having sixteen amino acids or less, in particular a hexapeptide, Y/CPQGNH maintain the mitogenic activity. The peptide AQPYPQGNHEASYG (14-Ser) is effective in reversing both nephrotoxic and ischemic acute renal failure in rats. The growth-promoting characteristics of the 22 and 45 kDa proteins and the peptides are useful in treating and diagnosing patients with kidney disease. Nucleotide sequences that encode the factor are useful to develop probes to locate similar factors, to identify genetic disorders involving the factor, and to produce the factor by genetic recombinant methods. The nucleotide sequences and fragments thereof, are also useful for diagnosis and treatment of kidney disorders.

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IN VITRO AND *IN VIVO* GROWTH-PROMOTING PROTEINS AND PEPTIDES FOR KIDNEY EPITHELIAL CELLS

BACKGROUND

Novel growth peptides derived from protein factors having molecular weights of about 22 and 45 kDa stimulate mitogenic activity of epithelial, but not fibroblastic cells, in particular, kidney epithelial cells in culture. These growth promoting effects were also demonstrated *in vivo*.

Acute renal failure is a serious disease associated with high mortality for which no "real" treatment currently exists. Acute renal failure is defined as the abrupt disruption of previously normal kidney function. It is caused by a wide variety of mechanisms including circulatory failure (shock), vascular blockade, glomerulonephritis, and obstruction to urine flow. In addition it can occur following surgery, trauma, sepsis, or with certain medications, particularly antibiotics and anticancer agents.

In 1985 some 140,000 Americans were hospitalized with acute renal failure (see 1990 National Institutes of Health Long Range Plan). The average cost of treatment associated with these cases was over $9000. Based on the growth in the disease over the past several years and normal inflation, it was estimated that currently some 240,000 patients develop acute renal failure annually at a cost of over $10,000 per patient. That translated to a staggering total cost to the U.S. healthcare system of almost $2.5 billion per year.

TABLE 1
AVERAGE COST PER HOSPITAL DISCHARGE FOR KIDNEY AND UROLOGIC DISEASES, UNITED STATES, 1985[1]

	Number of Discharges	Average Cost per Discharge
1. Acute renal failure	139,134	$9,329
2. Chronic renal failure	395,066	9,249
3. Kidney disease of diabetes mellitus	96,731	6,819
4. Kidney cancer	47,384	6,145
5. Hypertensive renal disease	182,625	5,796
6. Other intrinsic/systemic diseases	79,683	5,061
7. Bladder cancer	125,108	4,758
8. Impotence	30,452	4,344
9. Prostate cancer	246,201	3,791
10. Testicular cancer	14,219	3,711
11. Benign prostatic hyperplasia	482,348	3,648
12. Polycystic kidney disease	44,155	3,213
13. Glomerulonephritis	79,531	3,135
14. Bladder disorders	342,211	3,064
15. Urinary stone disease	453,018	2,920
16. Urinary tract infection	1,583,309	2,549
17. Incontinence	162,574	2,547
18. Hematuria	173,495	2,375
19. Prostatitis	108,024	2,010
20. Obstructive uropathy[2]	397,074	1,842
21. Other genitourinary infections	147,215	1,339
22. Preeclampsia	139,000	1,025
23. Testicular dysfunction	7,019	950

[1]Includes payments to physicians.
[2]Includes vesicoureteral reflux.

SOURCES: National Center for Health Statistics: National Hospital Discharge Survey, 1985 (all listed diagnoses). Department of Veterans Affairs, for year ending September 30, 1986 (first-listed diagnoses) (unpublished). Health Care Financing Administration, Medicare provider analyses and review data, 1985 (unpublished).

As can be seen in Table 1 from the 1990 National Institutes of Health Long Range Plan, kidney disease contributes to major medical costs in the United States, so factors reducing time to recovery, are beneficial to society.

Equally significant, is the fact that the number of cases of acute renal failure is growing at a rate of 9% per year (National Institutes of Health, 1995) and this

high rate of growth is expected to continue. A reason given for this rise in the incidence of renal failure is that "sicker" patients with a high risk of renal failure are surviving longer.

1. **Older patients**, who have a significantly higher incidence of acute renal failure (*e.g.*, patients over 65 are 5 times more likely to be hospitalized for acute renal failure than those ages 45 to 64) are now surviving serious medical incidents (*e.g.*, heart attack, stroke) as well as complicated surgery. Improved hospital intensive care units with more sophisticated monitoring and life support systems also aid in keeping "sicker" patients alive. In addition improved therapeutic agents for treating cancer and life-threatening infections are often nephrotoxic.

2. **Neonates**, who have an extremely high risk of kidney failure are also surviving at shorter terms and at significantly lower birth weights. Such infants formerly had difficulties overcoming severe lung and heart problems, but these problems can now be successfully treated with improved drugs and techniques, particularly in specialized neonatal intensive care units.

Because these advances in treatment modalities are expected to continue and even accelerate, it is likely that the number of cases of acute renal failure will continue to increase, perhaps at an even faster rate.

At the present time no real "cure" exists for acute renal failure. The current method of treatment is to "rest" the kidney by performing dialysis to correct metabolic imbalances and wait for kidney function to return spontaneously.

Dialysis is a technique in which impurities and toxins from the blood, that are normally cleared through the kidneys are artificially removed through an extra-corporeal circuit and filter (hemodialysis) or through the peritoneal membrane. By removing such impurities the life threatening metabolic imbalances resulting from kidney failure can be corrected and the patient stabilized.

Mortality rates resulting from a patient's developing acute renal failure are extremely high. A recent study (Levy *et al.*, 1996) that analyzed the effect of acute

renal failure on patient mortality cites such rates as ranging from 42% to 88% based on 18 previously published reports. These rates have remained essentially unchanged since the early 1950's. In the 1996 study itself the mortality rate for hospitalized patients who developed acute renal failure was 5 times higher compared to similar patients without renal failure (34% vs. 7%).

A key finding of this study is that "acute renal failure appears to increase the risk of developing severe non-renal complications that lead to death and should not be regarded as a treatable complication of serious illness." Thus it appears that the rapid reversal of acute renal failure can significantly reduce the risk of mortality in patients who also frequently have complicated clinical courses by preventing the development of severe and often fatal non-renal complications.

It has long been known that the kidney is one of the few human organs that has an ability to repair itself after injury. Even in cases where the kidney has been irreversibly damaged, and there is extensive necrosis of kidney cells, strong evidence exists that some new cell growth occurs.

It has been proposed that growth factors are a therapeutic approach to stimulate or augment the regenerative process in the injured kidney and thereby reduce the severity and shorten the course of acute renal failure. The use of growth factors as a treatment for acute renal failure was first proposed by Toback (1984). However, finding suitable growth factors proved difficult. The rationale for this strategy was subsequently expanded after several specific growth factor proteins were identified (Mordan and Toback, 1984; Toback 1992; Mendley and Toback, 1989; Toback 1992 a and b). However, no factors have yet been confirmed as useful in treating humans.

Growth factors acting *in vivo* to stimulate proliferation and migration of noninjured tubular cells in the kidney, and possibly to facilitate recovery of sublethally-injured cells as well, would be beneficial. A specific growth factor could be used in combination with sufficient nutrients, calories, and dialytic therapy to increase survival of patients with renal problems. For example, administration

of growth factors could (1) increase positive outcomes in patients with cadaveric renal transplants, a situation in which acute renal failure is associated with increased rejection, (2) shorten the duration of acute renal failure which would increase patient survival, and (3) reduce the number of days required for hemodialysis treatment during the renal failure syndrome.

Autocrine growth factors are produced locally by the same cells on which they act. They appear to be produced in response to a stimulating event such as cell injury. Moreover, they are produced in extremely small quantities and may exist at detectable levels for only a short time. Consequently, they have been quite difficult to isolate and identify.

Two other types of growth factors—paracrine and endocrine—both appear to have some role in stimulating kidney cell growth. Paracrine factors act on adjacent cells (rather than on the cells that produced them) while endocrine factors are produced in one cell and transported (*e.g.*, by the blood stream) to act on another, distant cell. Several of these types of factors, which are typically produced in larger quantities, and have a longer "half life" than some autocrine factors, have been discovered, and their cDNAs identified.

Animal and Clinical Studies

Several growth factors have been studied in an acute renal failure rat model to determine their efficacy in speeding recovery. The results of these studies give encouraging support to the theory that growth factors may play a major role in accelerating kidney repair. Three of the most important of these are:

1. **Epidermal growth factor (EGF)** has been reported to accelerate recovery in rats with acute renal failure (Coimbra et al., 1990). However, it was noted that EGF also mobilizes calcium from bone, which is a serious side effect that will likely prohibit its use in humans.

2. **Insulin-like growth factor-I (IGF-I).** Several studies in the rat model confirm that this factor is indeed efficacious. However, in two clinical

studies in humans IGF-1 did not appear to have any substantial effect in speeding a patient's recovery from acute renal failure.

3. **Osteogenic protein-1 (OP-1)** is a bone growth factor already approved for human use in repairing bone, cartilage, and eye tissue. Although OP-1 may play a key role in the embryonic development of human kidneys, it is not clear how it works to help repair adult kidney cells. It is possible that OP-1 and other autocrine kidney growth factors together could have complementary mechanisms of action.

Autocrine Kidney Growth Factors

Although the animal study results on the previously identified growth factors are encouraging, none of these factors are used clinically at present. Of particular note is that the kidney messenger RNA for the three growth factors described above—EGF, IGF-1 and OP-1-actually **decreases** in the kidneys during acute renal failure. Logically, if a growth factor is to be effective in repairing injury and reversing acute renal failure, its levels would be expected to **increase** during this clinical event.

Some of the factors cited above are released by kidney epithelial cells and are capable of stimulating growth of the cells in an autocrine manner. For example, monkey kidney (BSC-1) cells respond to culture medium with a reduced concentration of potassium by releasing the "Low Potassium Growth Factor," and respond to a reduced concentration of sodium by releasing the "Low Sodium Growth Factor" (Mordan and Toback, 1984; Walsh-Reitz *et al.*, 1986; Toback *et al.* 1992b and 1995).

A significant need exists for new therapeutic approaches to "cure," or at a minimum, speed the reversal of acute renal failure.

SUMMARY OF THE INVENTION

The invention includes the family of proteins and peptides[1] including those having amino acid sequences of novel mitogenic proteins termed Wound Growth Factor (WGF), and its NH_2- terminal peptides which also stimulate growth of kidney epithelial cells. "Bioactive WGF" is defined herein as a factor that stimulates mitogenic activity in cultured renal cells and is generally what is meant by "WGF" herein. "WGF" includes peptides of various lengths, derived from the parent molecule as long as they have mitogenic activity. Suitable "WGF-derived peptides" are those in which amino acid substitutions, additions or deletions occur, but the peptides stimulate mitogenic activity, and their action is blocked by the pentapeptide YPQGN, or any other peptide that blocks activity. It is believed that at least the pentapeptide YPQGN blocks access to a cellular WGF receptor. A preferred hexamer (6-mer) sequence, *NH_2*-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-*COOH* (Y/CPQGNH) is generally included in the peptides. (A slash between amino acids at a position indicates either one is present.)

The invention is directed to biological growth-promoting proteins and peptides derived from an amino acid sequence, initially referred to as characterizing a protein designated *Wound Growth Factor* (WGF) because of the manner of production in culture of the basic "factor". Further analysis initially revealed factors of two molecular weights,

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                          1                                12        16
22 kDa isoform: NH2- A Q P Y/C P Q G N H E A T  S S S F -COOH

                          1                             12    14
45 kDa isoform: NH2- A Q P Y/C P Q G N H E X  A/S Y G -COOH
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An embodiment of a novel peptide of the present invention derived from WGF is a potent mitogen for monkey kidney epithelial cells in culture, the peptide including a 14 amino acids sequence, AQPYPQGNHEASYG. This 14-amino acid

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[1] Generally "proteins" is the term used for molecules of about 50 amino acids or greater. Peptides are smaller.

peptide (14-mer) is called "14-Ser" to indicate that it has a serine residue at position 12. Compared to other known renal growth factor mitogens, this peptide containing the sequence YPQGNH or CPQGNH, or native full-length WGF has a mitogenic effect that is either additive with, equivalent to, or more potent than other known factors: *e.g.*, epidermal growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, insulin-like growth factor-I, vasopressin, or calf serum.

The naturally-occurring factor (WGF) is released into culture medium when a kidney cell monolayer is subjected to mechanical scrape wounding with a pipette tip. This was a novel finding. That is, the growth factor is released from scrape-wounded kidney epithelial cells and can stimulate proliferation of the same type of cells. Thus, it is an autocrine growth factor. A source of cells which releases the factor is the BSC-1 cell line (nontransformed African green monkey kidney epithelial cells) (*ATCC CCL 26/BS-C-1*). These peptides may also be synthesized by techniques well known to those of skill in the art, including peptide synthesis and recombinant genetic technology.

The appearance of growth-promoting activity after wounding is likely mediated by the proteolytic activation of an inactive precursor of WGF. Evidence for this is that preincubation of the cells for 10 minutes with each of the following diverse protease inhibitors prevented the appearance of growth-promoting activity after wounding: aprotinin, phenylmethylsulfonylfluoride (Sigma) (PMSF), antipain, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride (TLCK) or α_2-macroglobulin. None of these agents inhibited cell growth when added to cells of nonwounded cultures. When added to the medium after the appearance of WGF mitogenic activity, neither PMSF nor aprotinin appeared to inhibit the increment in cell proliferation. HPLC-purified WGF does not appear to be a protease because it did not exhibit proteolytic activity when assayed using Protease Substrate Gel tablets (BioRad).

As discussed herein, WGF exhibits a growth-promoting activity after it is released into the culture medium of scrape-wounded kidney epithelial cells such as

BSC-1 cells. Isolation and purification of components responsible for this growth-promoting activity revealed that the components behave on sodium dodecylsulfate (SDS)-polyacrylamide electrophoresis as if they had a relative molecular mass (M_r) of 22 and/or 45 kilodaltons (kDa). Further analysis revealed that WGF is a protein that is a mitogen for monkey kidney BSC-1 cells, but not for 3T3 fibroblasts. Release of WGF also appears to be relatively kidney epithelial cell-type specific in origin, because it appears after wounding BSC-1 cells in culture, but not after wounding fibroblasts in culture.

Therefore, an aspect of the present invention is a protein designated "WGF" having the following characteristics:

a) an estimated molecular weight of about 45 and/or 22 kDa, said estimate obtained by electrophoresing the HPLC-purified protein on an SDS-polyacrylamide gel;

b) capable of stimulating mitogenic activity when in contact with cultured cells; and

c) released by BSC-1 cells in culture by scrape wounding.

In particular, the 45 kDa protein isolated from its natural state has a partial amino acid sequence at its amino-terminal end as follows: *NH_2*-alanine-glutamine-proline-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-glutamic acid-X-alanine/serine-tyrosine-glycine-*COOH*. (X = undefined amino acid)

Another WGF protein has an estimated molecular weight of about 22 kDa, said estimate obtained by electrophoresing the HPLC-purified protein on an SDS-polyacrylamide gel and obtaining a partial amino acid sequence at its amino terminal end as follows: *NH_2*-alanine-glutamine-proline-tyrosine/cysteine- proline-glutamine-glycine-asparagine-histidine-glutamic acid-alanine- threonine-serine-serine-serine-phenylalinine-*COOH*.

A protocol suitable to purify WGF from conditioned cell culture medium utilizes ultrafiltration, heparin-affinity chromatography and reversed-phase (RP) high-performance liquid chromatography (HPLC). 6,400 fold purification is

achieved, although the yield of WGF protein is extremely low, usually in the range of about 50 ng protein per liter of conditioned medium.

The size of bioactive WGF was defined by electrophoresing HPLC-purified WGF on SDS gels in parallel with standard proteins (*i.e.*, proteins of known sizes), slicing the gel into 2-mm wide gel fragments, eluting each fragment in buffer, and then assaying the eluate for mitogenic activity using cultures of BSC-1 cells. This experimental strategy indicated that WGF proteins have an estimated M_r of 22 and 45 kDa and are mitogenic.

A single sharp peak of absorbing material at 214 nm obtained by RP-HPLC exhibited growth-promoting activity on kidney epithelial, but not fibroblastic cells, and yielded several bands on SDS-polyacrylamide gel electrophoresis following silver staining.

Amino acid compositional analysis of material that formed the sharp peak confirmed the protein character of WGF. Microsequencing revealed the first 16 amino acids of the amino NH_2-terminus of the 22 kDa isoform: *NH_2*-alanine-glutamine-proline-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-glutamic acid-alanine-threonine-serine-serine-serine-phenylalanine-COOH. For the 45 kDa isoform, 14 amino acids at the amino-terminus have been identified: *NH_2*-alanine-glutamine-proline-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-glutamic acid-X-alanine/serine-tyrosine-glycine-*COOH*. The identity of the amino acid at position 11 is uncertain (X), and it is not possible to determine whether a tyrosine (Y) or cysteine (C) is at position 4, or an alanine (A) or serine (S) is at position 12. A search of the seven peptide sequence databases in the Experimental GENINFO(R) BLAST Network Service (Blaster) operated by the National Center for Biotechnology Information (NCBI) indicated that the amino-terminal sequences are that of novel proteins.

Of substantial importance was the additional finding that peptides smaller than the 16 amino acid sequence also had strong mitogenic specific activity. This finding was quite significant because these small peptides: (1) are much less likely

to be antigenic (*i.e.*, they can be directly infused into another animal or human without being rejected by the immune system), and (2) they can be readily prepared in large quantities and modified using a peptide synthesizer without first having to find a cDNA clone that encodes the entire 22 or 45 kilodalton protein, and then expresses the recombinant protein.

In addition to producing the factor by wounding cultured cells, synthetic peptides were produced that express the mitogenic activity. Of particular interest is a synthetic peptide whose sequence is based on the first eleven amino acid residues of the 22 kDa protein and exhibits mitogenic activity. Moreover, other polypeptides that are short peptide domains of the factor are also within the scope of the present invention. An hexamer was the smallest peptide which still maintained mitogenic activity (YPQGNH or CPQGNH).

A peptide comprising an amino acid sequence of *NH_2*-tyrosine/ cysteine-proline-glutamine-glycine-asparagine-histidine-*COOH* is suitable for the practice of the invention. Generally, the peptide has a length of from 7 to 16 amino acids, but other lengths are also suitable if the mitogenic and/or antigenic function is preserved.

Both transforming growth factor-beta 2, and a synthetic 5-amino acid peptide having the sequence YPQGN block the mitogenic effect of AQPYPQGNHEASYG. The glycosaminoglycans, heparin and keratan sulfate, each potentiate the mitogenic effect of the peptide.

A peptide having the sequence AQPYPQGNHEASYG (14-Ser) and other related peptides derived from the NH_2-termini of Wound Growth Factor isoforms, were evaluated for their capacity to alter the course of acute renal failure (ARF) in both nephrotoxic and ischemic rat models; this syndrome commonly afflicts humans.

Mercuric chloride given subcutaneously (s.c.) was used to induce ARF in rats, and a solution of each peptide was evaluated for its capacity to enhance

survival and speed recovery of renal function, assessed by measuring the serum creatinine concentration during the ensuing 7 days.

Administration of a peptide (100 μg) having the sequence AQPYPQGNHEASYG, s.c., 1 hour after administration of mercuric chloride significantly improved recovery of renal function two days later and improved survival after three days. Beneficial effects of the peptide on survival and recovery of renal function were also observed when it was administered 16.5 hours after or 24 hours before induction of the nephrotoxic ARF syndrome.

In ischemic ARF induced in rats by 45 minutes of bilateral renal pedicle clamping, administration of AQPYPQGNHEASYG peptide (100 μg) 1 hour after removing the clamps also improved survival and enhanced recovery of kidney function.

Evidence that the peptide improved survival and recovery of renal function by stimulating DNA synthesis in cells near the site of mercuric chloride-induced renal injury was obtained by using bromodeoxyuridine to label DNA in the regenerating kidneys. The peptide was more potent than epidermal growth factor, an agent of known efficacy in this model system, in promoting survival when given 24 hours before renal injury, and equivalent when given shortly thereafter. Improved survival and a more rapid recovery of renal function in response to treatment with the peptide are expected in humans with nephrotoxic as well as ischemic ARF.

Suitable peptides for the practice of the invention include:

AQPY/CPQGNHEATSSSF;
AQPY/CPQGNHEATSSS;
AQPY/CPQGNHEA;
AQPY/CPQGNHEAT;
AQPY/CPQGNHEATS;
AQPY/CPQGNHEATSS;
AQPY/CPQGNHEAAYG;
AQPY/CPQGNHEAAY;
AQPY/CPQGNHEAA;
AQPY/CPQGNHE;
AQPY/CPQGNHEASYG;

AQPY/CPQGNHEASY;
AQPY/CPQGNHEAS;
QPY/CPQGNHEA;
AQPY/CPQGNH;
QPY/CPQGNHE;
PY/CPQGNHEA;
QPY/CPQGNH;
PY/CPQGNHE;
Y/CPQGNHEA;
PY/CPQGNH;
Y/CPQGNHE;
Y/CPQGNHEATSSSF;
Y/CPQGNHEATSSS;
Y/CPQGNHEATSS;
Y/CPQGNHEATS and
Y/CPQGNHEAT.

The peptides YPQGNH or CPQGNH (Y/CPQGNH) are each a mitogenic fragment of protein.

Variants (isoforms) of the various WGF-derived NH_2-terminal peptides or full-length WGF protein can differ in their amino acid sequence or in ways that do not involve sequence modifications such as post-translational modifications (*e.g.*, glycosylation), or both. Variants in amino acid sequence are generated when one or more amino acids of the NH_2-terminal peptides or WGF protein is substituted with a different naturally-occurring amino acid, an amino acid derivative, or non-native amino acid. Particularly preferred variants include native WGF protein isoforms, biologically-active peptides of naturally-occurring WGF protein, and synthetic peptides whose sequences differ from the wild type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Variants may also have sequences that differ by one or more non-conservative amino acid substitution, deletions or insertions that do not abolish the biological activity, that is, the mitogenic activity of the WGF peptides or full-length protein.

Functionally equivalent peptides may be constructed by substituting equivalent amino acids, and using the mitogenic assay to demonstrate that the

molecule produced stimulates growth of BSC-1 cells by 15 to 35% compared to a functionally inert control peptide. Peptides constructed with these "equivalent" or "conservative" substitutions may stimulate, inhibit, or have no effect of renal epithelial cell growth. A guide to "conservative substitutions" is presented in Table 2. Syntheses of peptides with substitutions is readily performed by those of skill in the art, and the mitogenic assay is easy to perform. Therefore, peptides within the scope of the invention are readily determined.

Conservative alterations typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following pairs or groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively-charged (basic) amino acids include arginine, lysine and histidine. The negatively-charged (acidic) amino acids include aspartic acid and glutamic acid.

Table 2 summarizes additional conservative substitutions; others have been described by Dayhoff in the *Atlas of Protein Sequence and Structure*.

TABLE 2:
SOME CONSERVATIVE AMINO ACID REPLACEMENTS

Amino Acid	Single Letter Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-cys, D-Cys
Arginine	R	D-Arg, Lys, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu

Amino Acid	Single Letter Code	Replace with any of
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

As described herein, analysis of chromatograms following microsequencing indicated that the identity of certain amino acid residues in the NH_2-termini of WGF isoforms was ambiguous. Using each of the plausible amino acids to prepare a specific synthetic peptide allowed identification of WGF-derived molecules with different mitogenic potencies (Table 3).

A composition comprising the proteins or peptides described herein is within the scope of the invention.

A method for producing a protein or peptide of the present invention includes the steps of:

a) culturing kidney epithelial cells in media,

b) scrape-wounding the cells in culture, and

c) obtaining the protein from the conditioned medium.

The protein obtained is isolated from the conditioned medium and purified. A source of the kidney epithelial cells in culture is the BSC-1 African green monkey kidney epithelial cell line.

A recombinant DNA method of making a protein or peptide of the present invention includes the following steps:

a) obtaining a nucleotide sequence encoding the protein or peptide; and

b) using the nucleotide sequence in a genetic expression system to make the protein or peptide.

The peptides of the present invention can also be prepared directly on a peptide synthesizer, without recourse to cellular or molecular biological techniques.

Antibodies to WGF and derived peptides is an aspect of the present invention. Availability of each antibody provides a diagnostic tool to measure the amount of the factor and its derived mitogenic peptides in urine, blood and tissue. New diagnostic insights are facilitated in patients receiving drugs with nephrotoxic potential during treatment of infections or malignancies, and in individuals with renal injury or neoplasia. Such an antibody may also be used to detect renal cancer in the remnant kidneys of patients undergoing chronic peritoneal dialysis and/or hemodialysis. The antibodies, are for example, directed to a protein or peptide including the active site, that generally includes Y/CPQGNH.

A diagnostic kit is used to measure the quantity of a WGF protein or a mitogenic peptide derived therefrom, in a biological sample to detect acute renal injury or the early onset of kidney disease, monitor treatment of renal cell cancer, or recognize the conversion of benign renal cysts in chronic dialysis patients to carcinomas or cystadenocarcinomas. The kit includes in separate containers:

a) an antibody to WGF or to a mitogenic peptide derived therefrom; and

b) a means for detecting a specific complex between the WGF protein or a mitogenic peptide, and the antibody.

Controls and buffers may also be included.

The invention includes the use of the peptides in preparing a composition for medical treatment of kidney disease, said preparation comprising obtaining the peptides and adding to them a suitable carrier. Such carriers are well-known in the art.

The new kidney growth factor proteins and peptides of the present invention, and antibodies directed to them, have diverse uses in clinical medicine. The WGF peptides are useful for stimulating kidney cell growth, a characteristic useful for treatment of acute renal failure. It is particularly desirable to speed recovery in patients with acute renal failure, especially those receiving cadaveric kidney transplants that have reduced growth potential. Infusion of the protein or peptides into patients is directed to shortening the duration of the acute renal failure episode which would increase patient survival, and reduce the number of days required for hemodialysis treatment during the renal failure syndrome. The peptides also provide an *in vitro* standard of comparison for other candidate growth factors. One or a plurality of peptides may be used.

It is expected that WGF will have a role as a therapeutic agent to slow the progression of established kidney diseases such as chronic glomerulonephritis or interstitial nephritis. WGF and its receptor appears to be on the surface of renal epithelial cells. If WGF is found to be a ligand for receptors on the surface of specific renal epithelial cell types along the nephron, it is to be considered in cancer chemotherapy. If it is found to be cancer cell-type specific, the growth factor could be conjugated with a cellular toxin, a radioactive isotope, or cytotoxic antibody to produce powerful new chemotherapeutic agents.

A method of treating a person with acute renal failure, includes: a) preparing a pharmacologically effective amount of native WGF protein or

WGF-derived peptide in a suitable diluent; and b) administering the preparation to the person. The WGF may be ligated to a cytolytic ligand, *e.g.* a toxin.

The invention also relates the use of a protein or peptide described herein to obtain a composition useful in treating a person with acute renal failure.

Multimers may be even more effective in increasing cellular mitogenic activity, *e.g.* as the 40-mer disclosed herein. Multimers may be formed from homogeneous peptides, *e.g.* multimers of the hexapeptide Y/CPQGNH, or may be combinations of peptides from the WGF. A composition may include at least one multimer of a WGF-derived peptide, made from one of the group consisting of the hexapeptide NH_2-Y/CPQGNH-*COOH*, or the first 10 amino acids of the 22-kD and 45-kD WGF protein (AQPYPQGNHE), or the 14-Ser peptide (AQPYPQGNHEASYG), or multimers of other WGF sequences, or combinations of any of the peptides disclosed herein.

The composition may include at least one WGF-derived peptide in which one or more amino acids are replaced with a naturally-occurring amino acid, an amino acid derivative, or a non-native amino acid, so long as the resulting variant retains its mitogenic activity. A suitable WGF-derived peptide may have an amino acid sequence that differs by at least one amino acid substitution, deletion, or insertion from another WGF-derived peptide having mitogenic activity.

Also within the scope of the invention are compositions including a structural homolog of WGF protein whose nucleotide and amino acid sequences have at least 80% homology and exhibit mitogenic activity.

Also included within the scope of the invention are compositions including at least one structurally altered isoform of WGF protein that is posttranslationally modified by one of the following: glycosylation, sulfation or myristilation, retain mitogenic activity.

A composition of the present invention may also include a cell surface receptor(s) to which native WGF protein and WGF-derived peptides bind to initiate mitogenic signal transduction.

A composition may include the WGF protein or WGF-derived peptides in combination with other known growth factors and/or nutrients.

The compositions of the present invention are useful for methods of treatment of kidney failure or dysfunction, by a) preparing a pharmacologically effective amount of native WGF protein or WGF-derived peptide in a suitable diluent, optionally, with other growth factors and/or nutrients; and b) administering an effective amount of the preparation to the person with the kidney problems.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates growth-promoting activity of wound growth factor-derived peptides on growth of BSC-1 cells.

FIG. 2A illustrates enhanced survival and FIG. 2B shows renal functional recovery of rats given a WGF-derived peptide (14-Ser) after mercuric chloride-induced acute tubular necrosis. (A: Values are means ± standard error. *, Student's *t*-test, $P<0.034$.) (B: Percent survival is calculated as the number of rats alive divided by total number alive and dead; *, chi squared, $P<0.050$.)

FIG. 3A illustrates enhanced survival and FIG. 3B renal functional recovery of rats given WGF-derived peptide (14-Ser) after ischemic acute renal failure induced by bilateral kidney pedicle clamping. (Percent survival is the number of rats alive divided by the total number alive and dead.) Values are means ± standard error. *, Student's *t*-test, $P<0.032$.)

DETAILED DESCRIPTION OF THE INVENTION

Production Of The Wound Growth Factor from Kidney Cell Cultures

Confluent monolayer cultures were mechanically scraped using a 200 µL pipet tip (Continental Laboratory Products, San Diego, CA). When observed under a microscope, this scraping did not appear to damage the cells, only to cause them to separate from each other and retract, leaving a narrow path in the culture dish that could be seen with the naked eye. This process of scraping the cells has been

termed "wounding" and has been used for many years as a model system to study repair of corneal abrasions of the eye (Joyce *et al.*, 1990). In effect, pressure is applied to cells sufficient to disrupt intercellular adhesion. To determine whether kidney epithelial cells released an autocrine factor into the medium after wounding, conditioned culture medium was removed from the culture dish after scrape-wounding a monolayer of BSC-1 cells. An aliquot was assayed for any growth-promoting activity on a nonwounded "detector" culture (1.2×10^6 cells per 55-mm dish).

Growth-Promoting Activity of the Factor

Growth-promoting activity in an aliquot of the conditioned medium was assayed by counting the number of cells in a detector culture four days later and comparing the number to that in a control culture to which an aliquot of medium from a nonwounded culture had been added. This strategy showed that wounded kidney epithelial cells of BSC-1 line released a growth-promoting activity that was initially termed "Wound Growth Factor." 3T3 fibroblasts did not produce a similar effect.

For the assay, cells were detached from the dish with a solution of trypsin and an aliquot was counted in a hemocytometer. Confluent cultures were used for this assay so that cells exposed to WGF had a cell count of 2.6×10^6 cells/culture, whereas cells treated with an aliquot of medium from a non-wounded culture had a count of 2.0×10^6 cells four days after the additions. Thus WGF stimulated cell proliferation by 30% in this assay. When high-density, quiescent cultures (3×10^6 cells/55-mm dish) were used to assay mitogenic activity by measuring [^{3}H]thymidine incorporation into DNA, WGF enhanced DNA synthesis about by 60%. To prepare WGF, cells can be scrape-wounded every other day, yet maximal activity will be released into the conditioned medium. WGF is mitogenic for both sparse and confluent cultures of BSC-1 cells, and is stable upon storage at 4°C to -40°C for many weeks.

Characterization of Wound Growth Factor Mitogenic Activity

After detection of growth-promoting activity in the conditioned medium of scrape-wounded cultures of BSC-1 cells, efforts to determine size and composition of the mitogenic factor were undertaken. Initially, filters having different molecular weight cut-offs were utilized to show that the growth-promoting activity passed through an Amicon YM membrane having a cut-off of 100 kDa, but was retained by a membrane with a cut-off of 30 kDa, suggesting the size of the mitogenic factor was greater that 30 kDa, but less than 100 kDa. These membranes provide only a very crude estimate of size of the mitogenic factor.

Further analysis strongly suggested that the activity was a protein because it could be destroyed by exposure to trypsin (100 μg/ml for 3 hours), dithiothreitol (65 mM for 1 hour), acetic acid (1 M for 5 hours), or by heat (70°C for 20 minutes).

Characterization of the net electrical charge on WGF was sought using DEAE and CM cellulose matrices (Pharmacia) that have different charges. Results indicated that WGF was cationic. Subsequent experiments indicated that it bound tightly to a heparin cartridge (Pharmacia Hi Trap Heparin) from which it could be eluted with 0.4 to 1.0 M sodium chloride.

Pentosan polysulfate (Sigma Chemical, St. Louis) which is known to block the activity of heparin-binding growth factors such as acidic and basic fibroblast growth factors, also blocked activity of WGF suggesting that it is a cationic molecule. Studies on a C_4-reversed-phase HPLC column (Vydac) revealed that the protein was markedly hydrophobic in that a concentration of 55% acetonitrile (J.T. Baker, HPLC grade) was required to elute WGF-growth-promoting activity from the column. WGF activity was also stable in 0.1% trifluoroacetic acid (TFA) (J.T. Baker, HPLC grade) and isopropanol.

Certain mitogenically-active fractions of WGF eluted from a C_4 reversed-phase HPLC column also bind to concanavalin A-Sepharose 4B (Pharmacia LKB), and are eluted by alpha-methyl mannoside, suggesting the factor

contains carbohydrate. Four fractions from the HPLC column that exhibited maximal growth-promoting activity, including the one that elutes at 55% acetonitrile designated as WGF, were each exposed to concanavalin A-Sepharose and subsequently to alpha-methyl mannoside. The eluate was then assayed for its growth-promoting activity. Fractions that eluted from the HPLC column at 55% and at 49% acetonitrile bound to concanavalin A-Sepharose, were eluted by alpha-methyl mannoside and were fully active. In contrast, fractions that eluted at 46% or 57% acetonitrile, or pure epidermal growth factor used as a control mitogen that is not a glycoprotein did not bind to concanavalin A. These results suggested that native WGF is a glycoprotein

WGF, Heparin, and Other Growth Factors

Although scrape wounding of a different type of cells, endothelial cells, releases acidic fibroblast growth factor (aFGF), characterization of kidney cell WGF indicated that WGF differed from aFGF. Evidence for this is as follows: (1) heparin (Sigma) augments the mitogenic activity of WGF but inhibits aFGF when each component is added to BSC-1 cells, (2) keratan sulfate (a glycosaminoglycan component of the extracellular matrix) stimulates mitogenic activity of WGF but has no effect on activity of aFGF, (3) the mitogenic effects of maximal concentrations of WGF and aFGF are additive, and (4) the size of WGF is about 45 kDa whereas aFGF is about 16 kDa.

WGF also differs from basic FGF (bFGF) in that (1) bFGF (Gibco/BRL) elutes from an heparin affinity column at 1.5 to 1.6 M NaCl whereas WGF elutes at 0.4 to 1.0 M NaCl, and (2) the mitogenic activity of bFGF is 90% inhibited by 55% acetonitrile/ 0.1% (TFA) which has no effect on the activity of WGF. Furthermore, aFGF and bFGF mRNA are not detected by Northern blotting of BSC-1 cells, indicating that genes encoding these growth factors are not expressed by the cells.

The mitogenic effects of maximal concentrations of partially-purified WGF and epidermal growth factor (EGF, Promega) are also additive, indicating that they

probably exert their mitogenic effects by different receptors and signaling pathways.

Treatment of a confluent monolayer of BSC-1 cells with the enzyme heparinase I (Sigma), but not heparinase III (Sigma), releases WGF mitogenic activity from cells into the culture media. However, this seems to deplete WGF, at least temporarily. When cells are first treated with heparinase I, rinsed, the media aspirated, and the monolayer is then scrape-wounded, no growth-promoting activity is detected in the conditioned media. These observations suggest a role for a heparin-like molecule such as a glycosaminoglycan that mediates the association of WGF to the plasma membrane from which it is released upon wounding or treatment with heparinase I.

If WGF resides on the cell surface, it appears to be protected from degradation by trypsin, possibly by carbohydrate residues linked to its protein backbone, and/or by associating with glycosaminoglycans adherent to the plasma membrane (glycocalyx). Evidence in favor of this formulation is derived from the following experiment. A cell monolayer was exposed to the proteolytic enzyme trypsin (1 or 10 μg/ml) for 10 minutes. The enzyme was previously shown to have the capacity to destroy WGF activity. Then soybean trypsin inhibitor (10 μg/ml) was added to neutralize the enzyme. When the cell monolayer was subsequently wounded, full biologically active WGF was released into the culture medium indicating that exogenous trypsin had not destroyed growth factor associated with the cells.

WGF does not appear to be stored by the cells in extracellular matrix (ECM). This conclusion is based on the following experiment. A confluent monolayer of BSC-1 cells is detached from ECM by addition of EGTA (J.T. Baker) to the medium, leaving a coating of ECM on the surface of the culture dish. Fresh medium was added to the dish and its ECM coating was then subjected to scrape-wounding. No mitogenic activity was detected in the conditioned medium, suggesting that the activity resided on or within the cells and not in the ECM.

The mitogenic potency of partially-purified WGF is equivalent to the mitogenic potency of about 5% calf serum, or 20 pg/ml aFGF, or 20 pg/ml basic FGF, or 15 ng/ml EGF, using assays disclosed herein.

Comparison of Known Growth Factors to WGF-Derived Peptide; Additive Effects

The mitogenic potency of the 14-amino acid peptide AQPYPQGNHEASYG having a serine residue at position 12 (14-Ser) was compared to growth factors and other mitogenic signals for monkey kidney epithelial cells of the BSC-1 line. The previously identified maximal mitogenic concentration of each growth-promoting signal was employed, and compared to the WGF-derived peptide at concentrations from 0 to 0.5 μg/ml.

The following peptide and ionic mitogenic signals were studied: EGF, 50 ng/ml; IGF-I, 50 ng/ml; acidic fibroblast growth factor (FGF-1), 100 pg/ml; basic fibroblast growth factor (FGF-2), 1,000 pg/ml; vasopressin, 75 pg/ml; calf serum (0-1%); high potassium medium (5 mM added KCl, final concentration 10.4 mM), high sodium medium (25 mM of NaCl added, final concentration 180 mM).

Nontransformed monkey kidney epithelial cells of the BSC-1 line were grown to confluence in Dulbecco's modified Eagle's medium containing 1% calf serum and 1.6 μM biotin in 55-mm culture dishes at 38°C in a CO_2 incubator. When cells achieved a density of about 10^6 per dish, the spent medium was removed and replaced with fresh medium containing 0.5% calf serum and the 14-amino acid peptide (0.5 μg/ml). Additions of each of the mitogenic signals at maximal concentration were made to the culture medium. Four days later the number of cells in each culture was counted in an hemocytometer. Values obtained were the mean of 3 separate cultures. Doubling the calf serum concentration from 0.5% to 1.0% did not significantly alter the growth of BSC-1 cells. Addition of the 14-Ser peptide (0.5 μg/ml) stimulated growth by 25-30% at each of these serum concentrations.

1. Comparisons with EGF, IGF-I, Acidic FGF, Basic FGF, Vasopressin

The "14-Ser peptide" AQPYPQGNHEASYG increased cell number by 25-30% compared to control at four days. EGF (50 ng/ml) alone stimulated growth by 31% in the absence of "the 14-Ser peptide," and by 61% in its presence, indicating that the two mitogens were additive.

IGF-I (50 ng/ml) stimulated growth by 20%; it was less potent than the 14-Ser peptide. When both were added a 38% stimulation was observed. Although less than the expected 45% if fully additive, the mitogenic effect of IGF-I appeared additive to that of the 14-Ser peptide.

Acidic FGF (100 pg/ml), basic FGF (1,000 pg/ml), and vasopressin (75 pg/ml) stimulated growth by 17%, 18%, and 14%, respectively, compared to the 14-Ser peptide which increased cell number by 25%. Each of these known peptide growth factors was additive with the 14-Ser peptide peptide; acidic FGF by 44%; basic FGF, 41%; and vasopressin, 36%.

In summary, the 14-Ser WGF-derived peptide is a potent mitogen because it is equivalent to, or more potent than, each of five known peptide growth factors for kidney epithelial cells. In addition, the growth-promoting effect of the 14-Ser peptide was additive with each of the five growth factors suggesting that it acts by a different receptor and/or signal transduction pathway than they do. Finally, the additive mitogenic effects suggest that the 14-Ser peptide, in combination with one or more of the known growth factors, could act *in vivo* to speed repair and regeneration of the injured kidney during acute renal failure.

2. High Potassium Medium, High Sodium Medium

Raising the potassium concentration from the control value of 5.4 mM to 10.4 mM, or the sodium concentration form 155 mM (control) to 180 mM by addition of the appropriate salt solution serves as a mitogenic signal for kidney epithelial cells.

When the potassium concentration of the culture medium was raised from 5.4 to 10.4 mM by adding a solution of potassium chloride, the cell number was increased by 18%. Addition of the 14-Ser peptide was expected to enhance growth by 43%; an increase of 35% was observed.

Raising the sodium concentration of the medium from 155 mM to 180 mM by addition of sodium chloride solution increased growth by 25%. When the 14-Ser peptide was added an increment in growth of 41% was observed, less than the predicted 50%.

In summary, each of the two ionic mitogenic signals were additive with the 14-Ser peptide mitogen.

3. Time Required for the Growth-Enhancing Effect of 14-Ser Peptide

To gain additional insight into the affinity of the 14-Ser WGF-derived peptide for the cell surface, experiments were performed to determine the minimum amount of time required for the ligand to be in contact with renal cells to irreversibly commit them to accelerated growth.

Confluent monolayers of BSC-1 cells are prepared as herein and exposed to the 14-Ser peptide (0.5 μg/ml) for different amounts of time (0 to 30 minutes). At the end of the defined exposure period, culture medium containing the mitogen was aspirated, the monolayer was rinsed to remove nonadherent peptide, and fresh medium was added. Four days later the number of cells in the culture was counted.

Exposure of cells to the 14-Ser peptide for 2 minutes was sufficient to commit them to maximal growth stimulation (29%); half-maximal stimulation was at 1 minute. The 40-amino acid peptide (see Table 3) behaved similarly. In contrast, the commitment time for EGF is 4 minutes, and for vasopressin it is 2 minutes. Thus the 14-Ser peptide apparently requires a remarkably short time (2 minutes) to irreversibly bind to the surface receptor and commit cells to accelerated growth. The time required is equivalent to or less than other known renal cell

mitogens (EGF, 4 minutes; vasopressin, 2 minutes; low sodium growth factor, 5 minutes; high potassium medium, 6 minutes; adenosine monophosphate, 14 hours).

Purification of WGF

A protocol to prepare one liter of WGF conditioned medium is described herein. The usual yield of WGF protein is about 50 ng per liter as estimated by SDS-polyacrylamide electrophoresis and amino acid compositional analysis.

Conditioned Media Preparation: 100 cultures of BSC-1 cells are grown to confluence (6-8 x 10^6 cells) on plastic tissue culture dishes (Nunc) having a diameter of 10 cm in Dulbecco's modified Eagle's medium containing 2% calf serum. The medium is aspirated, and the culture is rinsed with phosphate-buffered saline (PBS) (Sigma) solution to remove medium and serum. Then 10 ml of PBS is added to each dish in preparation for wounding. A total of 5 wounds to the cell monolayer are rapidly made by scraping a 200 µL plastic pipet tip across the surface of the dish from edge to edge. About ten minutes later (9.5 minutes is preferred) the conditioned buffer is decanted into a plastic Nalgene beaker.

Isolation and Purification of WGF: After conditioning, pooled conditioned buffer is sterile-filtered (CoStar, 0.2 µm pore size) to remove any debris or detached cells into silanized (Aquasil, Pierce) glass bottles (Wheaton). The sterile conditioned buffer is diafiltered, desalted, and concentrated using a YM-30 disc membrane (Amicon) at 4°C. The concentrated material is then sterile filtered (Millex HA, 0.45 Fm) at room temperature, lyophilized to further reduce the volume, loaded onto a 1 ml heparin-affinity cartridge and eluted with a solution of 1 M NaCl. The cartridge is first exposed to 0.4 M NaCl in 10 mM sodium phosphate (pH 7.4) to elute nonmitogenic material; the same buffer containing 1 M NaCl is then used to elute mitogenic activity from the cartridge. The eluate is desalted, and the volume is reduced using a Centricon 3 filter (Amicon) by centrifugation (7500 *g* for 2 hours at 4°C). The concentrate (300-400 µl) is loaded onto a C_4-reversed phase HPLC column (250 mm x 4.6 mm, particle size 5 µm) (Vydac), and is eluted with a gradient of acetonitrile (1-80%) in trifluoroacetic acid

(0.1%) (TFA) for 50 minutes using a Beckman Gold Chromatographic System. At least 5 different protein peaks (monitored by absorbance at 214 nm) that exhibit mitogenic activity can be readily identified. However, the peak that elutes at ~55% acetonitrile routinely exhibits the most mitogenic activity and reproducibility in different isolates. This eluate is collected by hand into a silanized Eppendorf tube, loaded onto a C_8 column (Vydac), and rechromatographed using the same acetonitrile/TFA gradient described above. Again the mitogenic activity elutes at ~55% acetonitrile. Growth-promoting activity and total protein content is monitored at each step during the purification process. Depending upon the number of liters of conditioned buffer processed, it may be necessary to run a second C_8 column to optimize separation of the peak of interest. This bioactive material is then reduced in volume using a vacuum concentrator (Savant), and subsequently loaded onto a 12.5% SDS gel for electrophoretic separation.

The M_r of WGF is estimated by comparison of its electrophoretic migration to that of standard proteins of known molecular size. Different proteins in the gel are visualized by staining with silver (BioRad), or after blotting onto a PVDF membrane (Millipore) and staining the blot with Coomassie blue dye (Gibco/BRL). More than one band usually appears. To determine which band represented the mitogenic form of WGF, an unstained nonreducing gel was sliced into 2-mm wide fragments after electrophoresis, and each was eluted for 18 hours with agitation at room temperature in PBS containing acetonitrile (3%) and bovine serum albumin (0.1%). The eluate of each fragment was added to a culture of BSC-1 cells to determine its capacity to stimulate cell growth. This experimental strategy revealed that the active WGF proteins had a M_r of 22 and 45 kDa.

WGF appears to be released from cells after wounding; it does not appear in the culture medium of nonwounded cultures. Evidence in support of this conclusion was obtained by the following experiment. Conditioned buffer (1550 ml) from scrape-wounded cultures was obtained as described above and resulted in a C_8-HPLC peak that eluted at 55% acetonitrile and exhibited growth-promoting

activity, whereas the same volume (1550 ml) of buffer exposed to nonwounded cultures subjected to the same purification protocol did not display this protein peak or mitogenic activity.

NH_2-Terminal Sequence of Wound Growth Factor

To obtain the amino acid sequence of the NH_2-terminus of WGF, 2.5 μg of C_8-purified protein was obtained and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel. The relative electrophoretic mobility of the purified protein was compared to that of standard proteins of known molecular size. After blotting onto a PVDF membrane (Millipore) and staining with Coomassie Blue dye, two bands were seen corresponding to sizes of 45 kDa and 22 kDa. They were subsequently cut out of the blot and then loaded onto an ABI microsequencer. The results of microsequencing are expressed using conventional abbreviations and symbols for the amino acids listed below:

A, Ala, alanine
C, Cys, cysteine
D, Asp, Aspartic acid
E, Glu, glutamic acid
F, Phe, phenylalanine
G, Gly, glycine
H, His, histidine
I, Ile, isoleucine
K, Lys, lysine
L, Leu, leucine
M, Met, methionine
N, Asn, asparagine
P, Pro, proline
Q, Gln, glutamine
R, Arg, arginine
S, Ser, serine
T, Thr, threonine
V, Val, valine
W, Trp, tryptophan
Y, Tyr, tyrosine
M-NH_2, methionine amide
X, identity not determined
pE, pyroglutamic acid

A total of four determinations of the amino-terminal sequence of WGF have been made on different batches of conditioned media. The first fourteen amino acids of the 45 kDa protein in the amino- to carboxy- orientation (*NH_2 ----->COOH*) are shown below wherein the numbers refer to positions using the first amino acid as number one:

```
          1                   5                  10     12     14
NH2 - A  Q  P  Y  P  Q  G  N  H  E  X  A/S  Y  G-COOH
```

Determination of the sequence of three different isolates of the 22 kDa protein are:

```
      1                5                10                16
NH2 - A  Q  P  Y  P  Q  G  N  H  E  A  T  S  S  S  F-COOH
NH2 - A  Q  P  Y  P  Q  G  N  H  E  A  T  S  S/Y - COOH
NH2 - A  Q  P  Y  P  Q  G  N  H  E  A  T - COOH
```

The longest is 16 amino acids.

Importantly, the first 10 amino acids are identical in each of the four sequence determinations, suggesting that the 22 kDa protein could be a fragment or breakdown product of the 45 kDa protein. However, the different sequences of amino acids 12 to 14 in the 22 and 45 kDa proteins suggest that they could represent two WGF isoforms.

The NH_2-Terminal Domain of WGF is Mitogenic For Kidney Epithelial Cells

A sequence of the eleven NH_2-terminal amino acids AQPYPQGNHEA (11-mer) of the 22 kDa protein was used to prepare a synthetic peptide linked to a branched lysine core. This multiple antigenic peptide system (MAPS) was used to immunize animals to prepare a polyclonal antibody that would recognize WGF. The MAPS protein has a polylysine backbone that is attached to a resin at one end and has four branches at the other; each branch is ligated to one molecule of the 11-mer peptide. Surprisingly, when tested for growth-promoting activity, the MAPS peptide stimulated DNA synthesis and proliferation of monkey kidney epithelial cells of the BSC-1 line. Its maximal growth-promoting effect was similar to that of native WGF. When compared to a MAPS protein prepared from a different 11-amino acid sequence and another one prepared using an unrelated 16 amino acid sequence, only the sequence based on WGF protein exhibited mitogenic activity. In subsequent experiments, it was shown that the 11-amino acid peptide, in the absence of the branched lysine core, stimulated proliferation of these kidney cells to the same extent as did purified WGF protein. Based on two cell types tested, the 11-mer WGF peptide stimulated mitogenic activity of renal epithelial cells but did not stimulate growth of murine 3T3 fibroblasts.

In addition, the growth-promoting effect of the 11-mer peptide and of wound conditioned buffer were not additive, suggesting that the 11-mer peptide stimulates cell proliferation by the same receptor and signaling pathway as does intact WGF. These unexpected results suggest that the 11 amino acids of the NH_2-terminus represent a mitogenic domain of WGF, although other growth-promoting domains may be contained in the native 45 kDa protein whose estimated length is about 400 amino acids.

FIG. 1 shows equivalent mitogenic dose responses of a synthetic 11-mer peptide, AQPYPQGNHEA which represents native (22 kDa) amino-terminal WGF, and the most active hexapeptide synthesized of the present invention YPEGNH, which differs from native WGF by replacement of a glutamine (Q) (charge=0) residue with glutamic acid (E) (charge=-1). However, the native sequence is preferred (see Table 3). The "native" sequence is that found in the 22 or 45 kDa WGF as obtained from cultured cells. The peptides are purified by reversed-phase HPLC on a C_{18} column using a gradient of acetonitrile (1-80%) in 0.1% trifluoroacetic acid, lyophilized, and then dissolved in tissue culture medium.

Nontransformed monkey kidney epithelial cells of the BSC-1 line were grown to confluence in Dulbecco's modified Eagle's medium containing 1% calf serum and 1.6 μM biotin at 38°C in a CO_2 incubator. When cells achieved a density of about 10^6 per dish, the spent medium was removed and replaced with fresh medium containing 0.5% calf serum and different amounts of the two peptides. Four days later the number of cells in each culture was counted. Each value is the mean of 3 separate cultures.

Mitogenicity of WGF-Derived Peptides

To delineate the sequence of the smallest peptide that could stimulate renal cell growth, synthetic peptides of different lengths were prepared (see below), and purified by reversed-phase HPLC on a C_{18} column. Mitogenic activity was assessed in a culture of BSC-1 cells by counting the number of cells after exposure

to a specified concentration (0.5 to 20 μg/ml) of peptide for four days, and comparing the result to growth in the absence of added peptide.

PEPTIDES

		1	5								10	
WGF	11-mer:		NH_2-A	Q	P	Y	P	Q	G	N	H	E A-*COOH*
	9-mer:			Q	P	Y	P	Q	G	N	H	E
	8-mer:				P	Y	P	Q	G	N	H	E
	7-mer:				P	Y	P	Q	G	N	H	
	7-mer:					Y	P	Q	G	N	H	E
	6-mer:					Y	P	Q	G	N	H	

Like the 11-mer, each of five shorter peptides, (6 to 9 amino acids long) stimulated renal cell growth maximally to the same extent (25-30%) as did native WGF.

The following peptides (4 to 6 amino acids long) represent domains of NH_2-terminal WGF that did **not** stimulate cell growth, as compared to the active 11-mer of line 1:

		1			5					10
WGF 11-mer:	NH_2-A	Q	P	Y	P	Q	G	N	H	E A-*COOH*
6-mer:		Q	P	Y	P	Q	G			
4-mer:							G	N	H	E
5-mer:					P	Q	G	N	H	
5-mer:				Y	P	Q	G	N		
5-mer:					P	Q	G	N	H	

The following 6-mer and 7-mer peptides whose sequences/differ from WGF also did **not** stimulate cell growth:

6-mer:	Y	P	R	G	N	H	
7-mer:	L	K	Y	S	G	Q	D

The results presented above indicate that maximal mitogenic activity of peptides based on the native sequence resides in a 6-mer whose sequence is **YPQGNH** or the equivalent because:

(1) peptides containing this sequence that are 7, 8, 9 or 11 amino acids long stimulate growth to the same extent,

(2) each of two 5-mers that lack one amino acid from either the NH_2- or -COOH terminus of the 6-mer are **not** mitogenic to the same extent,

(3) a 6-mer (YP**R**GNH) that differs from YPQGNH in only a single amino acid is not mitogenic to the same extent,

(4) another 6-mer (**QP**YPQG) that differs from YPQGNH in two amino acids is not mitogenic, and

(5) neither a 4-mer (GNHE) whose sequence is found in WGF, nor a 7-mer (LKYSGQD) having an unrelated sequence, is mitogenic.

Thus the NH_2-terminus of native WGF contains an hexapeptide sequence, *NH_2*-**tyrosine-proline-glutamine-glycine-asparagine-histidine**-*COOH*, that is mitogenic for renal epithelial cells, as exemplified by the BSC-1 line.

Peptides of various lengths are also within the scope of the present invention, as long as the mitogenic hexamer sequence or its equivalent is included. Peptides of various lengths may be used individually or combined in various ways for specific purposes. These possibilities include (*NH_2*-terminus----->*COOH* terminus):

AQPYPQGNHEATSSSF
AQPYPQGNHEATSSS
AQPYPQGNHEATSS
AQPYPQGNHEATS
AQPYPQGNHEAT

YPQGNHEATSSSF
YPQGNHEATSSS
YPQGNHEATSS
YPQGNHEATS
YPQGNHEAT

AQPYPQGNHEA
AQPYPQGNHE

QPYPQGNHEA
AQPYPQGNH
QPYPQGNHE
PYPQGNHEA
QPYPQGNH
PYPQGNHE
YPQGNHEA
PYPQGNH
YPQGNHE

These peptides may be combined with other amino acid sequences at either end or both ends (*NH_2*-and-*COOH*). The amino terminus Y may be substituted by cysteine.

Characterization of Native and Modified WGF-Derived Amino-Terminal Peptides

Amino acid replacements in the amino-terminus of native WGF were identified that could alter peptide mitogenic activity. The objective of this effort was to maximize the growth-promoting activity of the peptide for use in studies of its efficacy in treatment of acute renal failure in animal models of the syndrome. The strategy was to design and then chemically synthesize diverse peptides by substituting structurally related amino acids for those found in the native WGF sequence. The peptide of interest was then purified on a reversed-phase C_{18} column HPLC as described herein using a gradient of 1-80% acetonitrile in 0.1% trifluoroacetic acid (TFA). The purified peptide was then lyophilized to remove the acetonitrile and TFA and the lyophilized powder was weighed, dissolved in buffer and added to the culture medium to measure its effect on the growth of monkey kidney epithelial cells as described herein. To compare the relative potency of different peptides, only those molecules that stimulated growth maximally after 4 days in culture by 25-30% were analyzed further by defining their concentration-dependence. A value termed the $K_{½}$, was used. This term is defined herein as the peptide concentration in micromoles (μM) at which the growth-stimulating effect is one-half the concentration at which maximal

proliferation is observed. The lower the value, the greater the mitogenic potency of the peptide.

TABLE 3.

RELATIVE POTENCY OF WGF-DERIVED PEPTIDES ON GROWTH OF KIDNEY EPITHELIAL CELLS

PEPTIDE	AMINO ACID SEQUENCE	GROWTH-STIMULATION $K_{½}$, μM
14-mer	AQPYPQGNHEASYG	0.126
	AQPYPQGNHEASYG + keratan sulfate	0.067
	AQPYPQGNHEASYG + heparin	0.040
	AQPYPEGNHEASYG	0.128
	AQPYPQGNHEAAYG	0.199
	AQPYPQGNHEAAYG + keratan sulfate	0.054
11-mer:	YPQGNHEASYG	0.166
	YPQGNHEASYG + heparin	0.166
	AQPYPQGNHEA	0.260
	AQPYPEGNHEA	0.206
6-mers:	YPQGNH	0.352
	CPQGNH	0.361
	YPEGNH	0.301
	YPEGHH	0.472
	YPEGDH	11.03
	YPEGKH	14.10
	FPEGNH	2.72
	YPQGNH-amide	8.85
16-mer:	AQPYPQGNHEATSSSF	0.218
28-mer:	AQPYPQGNHEASYGAQPYPQGNHEASYG	0.067
	AQPYPQGNHEASYGAQPYPQGNHEASYG + keratan sulfate	0.042
	AQPYPQGNHEASYGAQPYPQGNHEASYG + heparin	0.013
40-mer:		
AQPYPQGNHEAQPYPQGNHEAQPYPQGNHEAQPYPQGNHE		0.012
AQPYPQGNHEAQPYPQGNHEAQPYPQGNHEAQPYPQGNHE + heparin		0.0051

Amino acids designated by capitals are those present in native WGF, whereas the bold letters refer to amino acid substitutions.

The results summarized in Table 3 indicate that in general, it is longer peptides (40-mer >28-mer >14-mer >11-mer > 6-mer) containing the sequence YPQGNH, that are the most potent. The longest, non-repeating, most potent peptide tested is the 14-mer AQPYPQGNHEASYG, *i.e.*, it has the lowest $K_{½}$, 0.126 μM. Note that exposure of the cells for 5 minutes to the glycosaminoglycan, keratan sulfate (2 μg/ml) prior to addition of the peptide, enhances the potency ($K_{½}$ = 0.067 μM) and heparin (1 μg/ml) is even more effective (0.040 μM). In rat studies the 14-mer was also effective in increasing survival of rats in which ARF was induced by mercuric chloride. The 14-mer with serine in position 12 was more effective than the 11-mer with glutamic acid in position 6.

The length of the 14-mer AQPYPQGNHEAAYG provoked interest about the three-dimensional structure of the peptide. Circular dichroism was carried out and revealed a spectrum consistent with a beta-pleated sheet conformation.

It is of interest that the potency of the 11-mer peptide AQPYPQGNHEA (native WGF sequence) is similar to that of the synthetic hexapeptide YPEGNH ($K_{½}$ ~0.3 μM) which has a single substitution: glutamic acid (E) replacing the native glutamine (Q). Therefore, some substitutions produce peptides with at least as effective mitogenic activity. However, certain conservative modifications of the 6-mer sequence result in drastic reductions in potency: replacement of the tyrosine (Y) residue with phenylalanine (F), substitution of asparagine (N) with aspartic acid (D) or lysine (K), or amidation of the carboxy-terminus.

Evidence to support the hypothesis that WGF and its peptides exert their mitogenic effect via a cell surface receptor mechanism was obtained by showing that pentameric peptides that lack one amino acid at either terminus of the hexapeptide YPQGNH, block the proliferative effect of intact hexamer. In these studies the pentamers, PQGNH or YPQGN were synthesized, purified by HPLC and then assayed for growth-promoting activity. Neither was mitogenic. When

either pentamer was added to the cell culture medium, it blocked the capacity of the hexamer YPQGNH added at the same time or afterwards to stimulate cell growth. These observations suggest that the pentamers and the hexapeptide compete for the same binding site/receptor on the plasma membrane. Because the pentamers are not mitogenic, when they bind to the site, growth is not observed. After pentamers are bound to the site, they appear to prevent the hexapeptide from gaining access to the membrane.

Additional evidence that the hexapeptides YPQGNH or YPEGNH have a high potency for renal epithelial cells was obtained by defining the time required for interaction between peptide and cells for irreversible commitment to accelerated proliferation. In these experiments a solution containing the peptide of interest was added to the cell monolayer; culture medium was then aspirated at a specified time thereafter. Then fresh medium without the peptide was added and the number of cells was counted four days later. Contact of the cells with the peptide YPQGNH for 2 minutes resulted in maximal growth-promoting activity, whereas the peptide could be completely washed off the cells at earlier times (0.5, 1, or 1.5 minutes) without stimulating growth. When the cells were exposed to keratan sulfate (2 μg/ml) for 5 minutes before adding the peptide, only 1 minute of exposure was necessary for the maximal mitogenic effect to be observed. Similar results were obtained when the peptide YPEGNH was tested; 3 minutes of exposure was sufficient to obtain a maximal growth response, but only 1.5 minutes when the cells were pretreated with keratan sulfate. Thus, keratan sulfate enhances the mitogenic potency of not only native WGF protein but WGF-derived peptides as well.

A search of the seven protein sequence databases (Blaster) operated by NCBI revealed that the NH_2-terminal sequence of WGF is novel and has limited homology with two known mitogenic proteins, gastrin releasing peptide (GRP) and bombesin, as shown below, using as the first position the first amino acid of the NH_2 terminus:

```
                    1        10         20      27
human GRP:          VPLPAGGGTVLTKMYPRGNHWAVGHLM-NH2
porcine GRP:        APVSVGGGTVLAKMYPRGNHWAVGHLM-NH2

                                  1   5    10    16
              WGF:  45 kDa        AQPYPQGNHEXAYG
                                              S
                    22 kDa        AQPYPQGNHEATSSSF

          bombesin:                pEQRLGNQWAVGHLM-NH2
                                    1         10  14
```

Symbols for amino acids in bold type represent identities between different proteins.

Alignment between the 14 amino acids of the NH_2-terminus of WGF and the bombesin molecule is limited to the consecutive **GN** residues (amino acids #7,8 in the 14 amino acid active peptide of WGF; #5,6 in bombesin), and an **A** residue (amino acid #11 in WGF; #9 in bombesin). Previous reports (Broccardo *et al.*, 1975; and Heimbrook *et al.*, 1988) indicate that the seven COOH-terminal amino acids of bombesin and of GRP are identical (WAVGHLM-amide), and are also the locus of mitogenic activity in these two proteins. A synthetic peptide comprising this sequence of amino acids is mitogenic for BSC-1 cells, but its $K_{½}$ is ~10 μM. Importantly, the mitogenic sequence of these seven COOH-terminal amino acids is **not** found in the mitogenic 16 amino acids of the NH_2-terminus of WGF.

The functional differences between WGF and other known sequences include that the hexapeptide **YPQGNH** is mitogenic for renal epithelial cells, whereas the GRP-derived hexapeptide **YPRGNH** is at most borderline mitogenic (10% stimulation) for renal epithelial cells and is not reported to be necessary for mitogenic stimulation of fibroblasts.

It is of interest that the mitogenic hexapeptide derived from the NH_2-terminus of WGF, **YPQGNH** (amino acids #4 to 9 in the WGF sequence), differs in only a single amino acid (**Q-->R**) from an hexapeptide sequence in human and porcine GRP, **YPRGNH** (amino acids #15 to 20), a domain that is not known to be mitogenic (Heimbrook *et al.*, 1988). There is no prior teaching or

suggestion to make an amino acid substitution at this position, no suggestion to make the particular substitution that is in WGF, nor any such a suggestion that substitution would confer mitogenic-stimulating activity on WGF.

These results indicate that the WGF-derived hexapeptide, YPQGNH, is a novel mitogen for renal epithelial cells.

Replacement of Tyrosine with Cysteine in WGF-Derived Hexapeptide Preserves Mitogenic Activity

The shortest synthetic peptide with mitogenic activity was initially found to have the sequence *NH_2*- tyrosine-proline-glutamine-glycine-asparagine-histidine-*COOH* (YPQGNH). This hexapeptide represents amino acids in positions #4 to #9 of the amino-terminus of WGF. The amino acid identified in position #4 of each of the two WGF isoforms by microsequencing could be either a cysteine (C) or a tyrosine (Y) residue.

The half-maximal concentration ($K_{½}$) at which the YPQGNH peptide exerts its growth-stimulatory effect is 0.35 μM (Table 3). Another synthetic hexamer (YPEGNH), in which glutamic acid (E) was substituted for the glutamine (Q) found in native WGF, was slightly more potent; its $K_{½}$ is 0.30 μM. To determine if cysteine could replace tyrosine in the mitogenic peptide, the hexapeptide CPEGNH was synthesized. This cysteine-containing peptide was indeed mitogenic, and its potency ($K_{½}$) was equal to that of the tyrosine-containing peptide. Support for the assertion that the amino acid in this position may be either a tyrosine or cysteine for maximal growth-promoting activity was obtained by analyzing the results of two additional experiments. In the first one, an hexameric peptide containing a phosphotyrosine (***Pi*-Y**) residue was synthesized with the sequence ***Pi*-YPQGNH**. In the second experiment, a peptide in which phenylalanine (F) replaced the tyrosine residue was synthesized to prepare FPEGNH. Importantly, the $K_{½}$ for *Pi*-YPQGNH was similar to that for YPQGNH (0.35 μM), whereas replacement of tyrosine with phenylalanine reduced the mitogenic potency of the peptide about 9-fold ($K_{½}$ for FPEGNH = 2.75 μM; $K_{½}$ for YPEGNH = 0.30 μM). Thus, peptide

mitogenic activity is not significantly affected when the hydroxyl group of tyrosine is phosphorylated, but is dramatically reduced when the hydroxyl group is absent (substituting phenylalanine for tyrosine).

These observations suggest that a sulfhydryl or hydroxyl group rather than the benzene ring of tyrosine in the amino acid residue confers mitogenicity on the hexapeptide. This analysis predicts that replacing tyrosine or cysteine with the hydroxyl-containing, naturally-occurring amino acids serine or threonine might also yield growth-promoting hexapeptides.

In summary, a tyrosine or cysteine residue in the hexameric cassette (Y/CPQGNH) of WGF-derived synthetic peptides of different lengths (*e.g.*, 6 to 40 residues) described herein, can fully support mitogenic function in kidney epithelial cells. Synthetic peptides that increase mitotic activity in an equivalent position containing either of these two amino acids, or others (Table 2), are produced and used to improve survival and speed functional recovery after acute renal failure.

Further Characterization of Mitogenic Peptides Derived From the NH_2-Terminus of Wound Growth Factor

As discussed in previous sections, purification of the Wound Growth Factor (WGF) from conditioned medium of scrape-wounded monkey kidney epithelial cells (BSC-1 line) revealed two mitogenic proteins; 22 kDa and 45 kDa on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Micro-sequencing of the NH_2-termini of these proteins suggested that they may be isoforms because the first 10 amino acids of each were identical. A total of 16 amino acids were identified at the NH_2-terminus of the 22 kDa isoform, and 14 for the 45 kDa isoform. However, for the 45 kDa isoform, the amino acid at position 11 was not identified, and at position 12, alanine or serine were equivalent designations. At position 4, tyrosine or cysteine were equivalent designations.

	1										12		16
22 kDa isoform:	NH_2-A	Q	P	Y/C	P	Q	G	N	H	E	A	T/S	S S F-COOH

1 10 12 14
45 kDa isoform: NH_2- A Q P Y/C P Q G N H E X A/S Y G -COOH

Peptides of various lengths are within the scope of the present invention, as long as the mitogenic hexamer sequence, *NH_2*-tyrosine-proline-glutamine-glycine-asparagine-histidine-*COOH* (YPQGNH) is included.

Amino acid replacements were identified in the amino-terminus of native WGF that could maximize the growth-promoting activity of the peptide for use in studies of its efficacy in treatment of acute renal failure in a rat model of this syndrome. The strategy was to design and then chemically synthesize diverse peptides by substituting structurally related amino acids for those found in the native WGF sequence.

The peptide of interest was then purified on a reversed-phase C_{18} HPLC column using a gradient of 1-80% acetonitrile in 0.1% trifluoroacetic acid (TFA). The purified peptide was then lyophilized to remove the acetonitrile and TFA and the lyophilized powder was weighed, dissolved in buffer and added to the tissue culture medium to measure its effect on growth of monkey kidney epithelial cells.

To compare the relative potency of different peptides, only those molecules that stimulated growth maximally after 4 days in culture by 25-30% were analyzed further by defining their concentration-dependence. A value termed the $K_{½}$, was used. This term is defined herein as the peptide concentration in micromoles (μM) at which the growth-stimulating effect is one-half the concentration at which maximal proliferation is observed. The lower the value, the greater the mitogenic potency of the peptide.

Table 3 indicates that the 14-amino acid peptide AQPYPQGNHEASYG with a serine residue at position 12 (14-Ser) ($K_{½}$=0.126 μM) is a more potent mitogen than AQPYPQGNHEAAYG having an alanine residue in that position ($K_{½}$=0.199 μM). When the glycosaminoglycan keratan sulfate was added at a concentration of 10 μg/ml the mitogenic potency of 14-Ser was enhanced because the $K_{½}$ fell to 0.067 μM. In addition, the glycosaminoglycan heparin, added at a

concentration of 1 μg/ml was even more effective, reducing the $K_{½}$ to 0.040 μM. At the optimal molar concentrations employed in these experiments, a molecular ratio of 1 peptide molecule: 1 glycosaminoglycan (GAG) molecule was added to the cells for either keratan sulfate or heparin. The stimulatory effect of glycosaminoglycans was mediated by formation of a peptide-GAG complex via ionic bonds (cationic histidine of peptide to anionic sulfate of either GAG) or by hydrogen bonding (hydroxyl groups of serine/tyrosine of peptide to oxygen residue of GAG carbohydrate). The peptide-GAG complex could stabilize/facilitate binding of the peptide to its receptor on the cell surface, as has been proposed to explain the enhanced mitogenic effect of acidic fibroblast growth factor (FGF-1) with heparin that has been observed with diverse types of cells. Replacement of a glutamine (Q) residue in position 6 with a glutamic acid residue (E) did not alter mitogenic potency of 14-Ser peptide ($K_{½}$=0.128 μM), although this replacement strategy does reduce the $K_{½}$ for the 6-amino acid peptide YPQGNH ($K_{½}$=0.352 to 0.301 μM), and for the 11-amino acid peptide AQPYPQGNHEA ($K_{½}$=0.260 to 0.206 μM).

Shorter peptides of 11 amino acids in length such as AQPYPQGNHEA ($K_{½}$=0.26 μM), and 6 amino acids, YPQGNH ($K_{½}$=0.35 μM) each lack the SYG terminal amino acids of the 14-amino acid peptide AQPYPQGNHEASYG ($K_{½}$=0.126 μM), and are less potent as mitogens. To determine if an 11 amino acid peptide containing this SYG sequence was required for maximal mitogenesis, an 11-mer peptide with the sequence YPQGNHEASYG was synthesized, purified, and then assayed for activity. Interestingly its $K_{½}$ was 0.166 μM in the absence or presence of heparin, indicating that the full 14 amino acid length was required for the maximal mitogenic effect.

To determine if lengthening the 14-Ser peptide would increase mitogenic potency, a dimer of the peptide having the sequence AQPYPQGNHEASYGAQPYPQGNHEASYG (28-mer) was synthesized, purified, and assayed for growth-promoting activity. The $K_{½}$ of the 28-mer was

0.067 μM, indicating it has twice the potency of 14-Ser whose $K_{½}$ was 0.126 μM (Table 3). Increasing peptide length is not the only mechanism to increase mitogenic potency, because the $K_{½}$ of 14-Ser in the presence of the glycosaminoglycan keratan sulfate (0.067 μM) (Table 3), is identical to the $K_{½}$ of the 28-mer.

A peptide synthesized using the longest known WGF sequence, the 16 amino acids of the 22 kDa isoform, AQPYPQGNHEATSSSF, was also tested for its mitogenic activity ($K_{½}$= 0.218 μM). It was not as potent as the 14-amino acid peptide AQPYPQGNHEASYG derived from the NH_2-terminus of the 45 kDa isoform (Table 3).

Finally, a 40-amino acid peptide was synthesized. It is a tetramer of the first 10 amino acids which are identical in the 22 kDa and the 45 kDa isoforms of WGF. This is the most potent WGF peptide yet identified, having a $K_{½}$= 0.012 μM. The enhanced mitogenic potency is presumably related to its relatively great length which could stabilize ligand binding at the cell surface receptor site by ionic or hydrophobic interactions, and/or hydrogen bonding. Ultimately, it may prove the most efficacious WGF-derived peptide under clinical conditions because of its great potency. Note that addition of heparin reduces the $K_{1/2}$ of the 40-mer to 0.0051 μM, making this the most potent WGF-derived peptide yet identified.

The 14-amino acid peptide (14-Ser), because of its high mitogenic potency and relatively short length, was chosen for use in the treatment of rats with mercuric chloride-induced acute renal failure.

An Antibody to Peptides Derived from WGF

The 11 amino acids of the NH_2-terminus of WGF were used to prepare two different conjugated peptide antigens. One was the MAPS protein described above in which the 11-mer peptide was linked to a branched polylysine backbone, and the other was a conjugate in which the 11-mer was chemically joined to keyhole limpet hemocyanin (KLH). The MAPS protein and KLH-conjugate were used to immunize rabbits, chickens, and a sheep. The antibody that results from these strategies serves to detect native WGF protein in urine, serum, and tissue in

patients, with acute or chronic kidney diseases, and renal cell cancer. It could also be used to obtain a cDNA clone encoding WGF by immunoscreening a kidney epithelial cell cDNA library in λgt11.

Lack of immunogenicity of the peptide would favor its clinical utility in the treatment of renal diseases such as acute renal failure.

Production of WGF by Recombinant Genetic Methods

An appropriate vector to express WGF from the nucleotide sequence encoding a full-length clone depends in part upon whether glycosylation of the protein is required for each of its biological effects. If WGF is not glycosylated, a bacterial expression system may prove suitable, but if carbohydrate is definitively detected and shown to be required for full activity, then a mammalian expression system such as Chinese Hamster Ovary (CHO) cells would be more appropriate.

Because the synthetic 14-mer peptide, indeed even an hexamer of the amino-terminus is mitogenic, it appears that glycosylation of the protein is not required for mitogenic activity. However, glycosylation could prove to be an important determinant in modulating the turnover (half-life) of the protein *in vivo*. This is the case for erythropoietin, for example.

A nucleotide sequence encoding for at least a mitogenic hexamer of the present invention is prepared, linked to suitable regulatory elements, and incorporated into an expression vector. A host cell is transformed with the vector, and the host is placed in conditions suitable for expression. After expression of the WGF gene or partial nucleotide sequence in a recombinant host, the recombinant protein is isolated from the culture medium by using an antibody-affinity column prepared by conjugating an antibody to WGF to an appropriate matrix. This preparative strategy should yield large quantities of the recombinant protein.

Alternatively, and more simply, sufficient amounts of the NH_2-terminal 14-Ser, 11 amino acid peptide, the hexapeptide Y/CPQGNH, or other peptides expressing equivalent mitogenic potency, can be prepared by conventional peptide synthesis. The mitogenic potency of the peptides prepared by recombinant or

chemical synthetic strategies can be assessed by measuring the capacity of an aliquot of the product to stimulate growth of BSC-1 cells. The expected response is a 20 to 35% increase in cell number after 4 days in confluent cultures compared to that observed in control cultures.

Detailed techniques to produce WGF by recombinant systems are known to those of skill of art.

In Vitro and *in Vivo* Models

In vitro and *in vivo* models of acute renal failure are useful to study two major biological characteristics of wound repair: cell migration and proliferation. These models are suitable for analyzing the effects of WGF and its peptides of the present invention.

i. ***In Vitro* model:** scrape-wounding of monolayer cultures of renal epithelial cells to simulate injured renal tubular epithelium.

In this model, high-density, quiescent monkey kidney epithelial monolayer cultures (BSC-1 line) are wounded by mechanically scraping away defined regions of the monolayer to simulate the effect of cell loss after tubular necrosis. The number of cells that migrate into the denuded area is counted (Kartha and Toback, 1992). It was found that cell migration is independent of cell proliferation, although both processes can be studied in this experimental preparation.

The model is useful to study the kinetics of renal epithelial cell migration, and identify genes whose expression is induced or repressed after wounding. The biological characteristics and potency of the WGF is suitable for investigations using this system.

ii. ***In Vivo* model:** mercuric chloride (nephrotoxic) and renal pedicle clamping (ischemic) models of acute renal failure (ARF) in the rat.

Previous studies using these model systems have characterized the clinical, histological, biochemical, and functional correlates of the ARF syndrome. It was demonstrated that infusion of a mixture of essential and nonessential amino acids stimulates synthesis of phosphatidylcholine and protein in regenerating rat renal

tissue and reduces the level of kidney dysfunction after intravenous administration of mercuric chloride (Toback, 1980). These studies showed that biochemical and functional repair of the injured kidney are not optimal in untreated animals and that provision of exogenous amino acids with glucose could speed recovery. The capacity of the WGF to enhance regeneration after ARF is studied in both the nephrotoxic and ischemic models of human ARF.

It is important to define the onset of abnormal kidney function and structure, its course, and subsequent recovery. In humans, as in rats, kidney function during ARF is monitored by measurements of the concentration of serum creatinine and blood urea nitrogen (BUN) on a daily basis. These measurements provide estimates of glomerular filtration in the kidney which is one of its major functions. Creatinine enters the serum from muscle where it is released constantly as a consequence of the metabolic turnover of creatine phosphate, whereas the BUN is the end-product of total body protein turnover. Both molecules make their way into the blood and because of their small size are excreted in the urine. Thus the extent to which they accumulate in the blood and are not excreted in ARF provides a guide to the extent of kidney injury. Significant recovery of kidney function is indicated by a reduction in serum creatinine concentration.

Treatment of Chronic Renal Diseases

The mechanisms that mediate the progression of renal disease in diabetic nephropathy, interstitial nephritis, and chronic glomerulonephritis are unknown. Relentless loss of renal function results in the need for chronic dialysis treatment at a cost of billions of dollars each year in the United States because at present there is no therapeutic strategy to slow or reverse the process. Patients early in the course of renal disease are suitable candidates for growth factor therapy because no other alternative is now available to treat this condition. The objective of administration of WGF compositions of the present invention is to speed recovery of injured renal epithelial cells along the nephron thereby repairing the damage mediated by the disease process, preserving renal function, and forestalling the need for dialysis.

Treatment of Renal Cell Cancer

Renal cell cancer is often a slow growing condition that results in widespread metastases to distant sites in the body making successful treatment difficult. Studies using radioactive WGF permit its receptor on renal cells to be identified and isolated; antibodies directed against the receptor are then prepared. These antibodies serve to localize receptors on specific types of kidney cells along the nephron. Armed with this information about the cell-type specificity of WGF receptors, strategies to treat cancers of renal cell types of interest include WGF or a specific WGF peptide that exhibits binding to the receptor and can thereby be used to deliver an anticancer agent to the proliferating cells of interest. Such anticancer agents comprise WGF or a specific WGF peptide or peptides conjugated to a toxin, cytolytic antibody, or a radioisotope. Although treatment of the primary renal tumor is likely best carried out by surgical extirpation of the organ, treatment of cancer metastases, especially those of small size, could be important targets of these novel chemotherapeutic agents that would be formulated using knowledge of WGF structure and function.

Delivery of WGF to Patients

Delivery to patients is generally by intravenous infusion similar to cancer chemotherapy deliveries or experimental treatment of small mammals. Another route is that used for treatment of anemia with erythropoietin (EPO) in renal failure wherein EPO is delivered every few days by subcutaneous injection.

For example, a typical dose of 14-Ser peptide for a 70-kg patient with ARF would be about 35 mg (100 μg per 200 gm body weight), administered by slow intravenous infusion.

Diagnosis of Renal Cell Injury by Use of an Antibody to WGF

The antibody to native WGF is prepared as disclosed herein and used to determine the concentration of the growth factor in urine and blood. Distribution of WGF in different types of renal cells along the nephron is determined. The

concentration of the growth factor in either blood or urine or both could increase as a result of renal injury. If the growth factor is excreted in the urine as is epidermal growth factor, it may be possible to determine if renal injury leads to increased urinary excretion of the new factors. If so, enzyme-linked immunosorbent assay (ELISA) kits to detect WGF are used to rapidly diagnose early renal injury prior to a fall in glomerular filtration rate which is not detectable with conventional laboratory tests until more than 50% of kidney function is lost. This is particularly valuable in patients receiving drugs with nephrotoxic potential during treatment of severe infections or malignancies. An antibody to WGF is incorporated into a diagnostic ELISA kit designed to detect the appearance of growth factor in blood and urine of patients with renal injury or neoplasia.

Detection of Renal Cancer in Chronic Hemodialysis Patients

If renal adenomas, cystadenomas, or carcinomas that occur in remnant kidneys of patients undergoing chronic hemodialysis are found to overexpress the growth factor and excrete it in the urine, an ELISA kit could provide a new early detection system for these lesions. Such a diagnostic kit could also be used in asymptomatic but high-risk individuals. Renal cancer is now difficult to detect early because it tends to be asymptomatic until the tumor has grown to significant size or has metastasized widely.

Manipulations of Growth Factor Structure and Functions

Analogs of the growth factor are developed to maximize the growth-promoting effect of WGF by optimizing specific binding to renal epithelial cell receptors. Inhibitors that block the biological action of each factor by binding to receptors on the cell surface are also useful. Such inhibitors include pentapeptides such as YPQGN and PQGNH. Development of a synthetic or recombinant product that is resistant to degradation would prolong pharmacological activity *in vivo*.

EXAMPLES

The following examples are provided for illustration, not limitation.

Example 1: 14-Ser Peptide is Effective in the Treatment of Nephrotoxic Acute Renal Failure

Nephrotoxic and ischemic acute renal failure (ARF) induced experimentally in rats have long been used to model this syndrome in humans. To determine if the mitogenic effect of 14-Ser, a WGF-derived NH_2-terminal peptide, would prove efficacious as a therapeutic agent in animals with ARF, acute tubular necrosis was induced by injecting a solution of mercuric chloride (in normal saline) subcutaneously into rats at a dose of 2.25 mg per kilogram body weight. Male rats weighed 200-225 gm at the start of the experiment. This dose was used because preliminary experiments indicated it resulted in survival of 25-50% of the animals 7 days later. Blood was obtained from the tail vein each day and the concentration of creatinine in the serum was measured and used as an index of renal function. An increase in serum creatinine concentration signals a decline in renal function because the injured kidney is unable to excrete endogenous creatinine in the urine so it accumulates in the blood.

The synthetic peptide AQPYPQGNHEASYG (14-Ser) (dissolved in sterile 0.01% bovine serum albumin in phosphate-buffered saline) was injected subcutaneously into rats to determine its effect on survival, recovery of renal function, and stimulation of DNA synthesis. Multiple different peptides were studied including 14-Ser, AQPYPEGNHEASYG (14-mer), AQPYPQGNHEATSSSF (16-mer), AQPYPQGNHEA (11-mer), and AQPYPEQGNHEA (11-mer). Different concentrations and times of administration were tested and compared. The results indicated that 14-Ser was the most effective WGF-derived peptide; it improved survival and recovery of renal function after mercuric chloride-induced ARF.

Acute tubular necrosis was induced in 44 rats by the subcutaneous (s.c.) injection of mercuric chloride, and a single dose of 14-Ser was administered s.c. 1

hour afterwards. Different amounts of the peptide were given to assess its capacity to affect the outcome. Survival 7 days later was about twice as high in animals given 100-150 μg of peptide (63%) than in rats given 0-75 μg peptide (29%) (chi squared, P = 0.015).

To determine the effect of the peptide on survival and recovery of renal function, acute tubular necrosis was induced in 59 rats; 29 received 14-Ser (100 μg/rat) and 30 received an equal volume of the vehicle s.c. 1 hour after administration of mercuric chloride. Enhanced survival (FIG. 2A) and renal functional recovery (FIG. 2B) of rats given a WGF-derived peptide after mercuric chloride-induced acute tubular necrosis is shown. Mercuric chloride was injected subcutaneously into each of the 59 rats, and 1 hour later 100 μg of WGF peptide (14-Ser) (n=29 rats) or the vehicle (n=30) was given. Survival was greater in rats given the peptide than in animals receiving the vehicle as early as *day 3* after administration of mercuric chloride. Blood was obtained from the tail vein on the days indicated, and serum creatinine concentration was measured. A similar increase in creatinine concentration was observed in both groups of animals on *day 1*, signifying that they sustained the same extent of renal functional injury. On *days 2, 3 and 4*, the lower creatinine concentration in peptide-treated rats indicates accelerated recovery of kidney function.

FIG. 2A indicates that the survival of rats given the peptide was significantly greater (chi squared, P=0.035) than those given vehicle 3 days after the onset of the ARF syndrome, a difference that persisted during the next 4 days. By day 7 only 20% of rats that received the vehicle were alive, whereas 48% of rats given the peptide survived (chi squared, P=0.012).

Assessment of renal function during the onset of the ARF syndrome revealed impaired function on *day 1* when the creatinine concentration increased from the basal value of 0.5 mg/dL to 2.3 mg/dL. It was similar in animals treated with the peptide (n=29 rats) or the vehicle alone (n=30) (FIG. 2B), suggesting that the extent of renal injury was equivalent in both peptide-treated and untreated

animals. On *day 2* the serum creatinine concentration was significantly lower in peptide-treated rats (n=29) than in animals given the vehicle (n=30) (P=0.034, Student's *t*-test), indicating that the decline in renal function was less severe in rats given mercuric chloride followed by the peptide. Evidence of a significantly more rapid recovery of renal function was also apparent on *days 3 and 4* after the onset of ARF. Similar beneficial effect of peptide administration was observed when the blood urea nitrogen concentration was measured as an index of renal function. These results indicate that a WGF peptide that is mitogenic for renal epithelial cells in culture can speed renal functional recovery and enhance survival of rats with nephrotoxic ARF.

Example 2: ***In Vivo* Administration of 14-Ser peptide at 16.5 hours after induction of nephrotoxic ARF.**

Because physicians are unable to predict with certainty when acute renal failure will occur in patients, the serum creatinine concentration is measured to determine if kidney function has been compromised. After renal dysfunction is detected, treatment to reverse the condition is highly desirable. Thus, the utility of administered 14-Ser peptide in human ARF requires that it act favorably when impaired kidney function has been detected. The peptide increased the speed of recovery of renal function when administered to rats after the onset of nephrotoxic ARF under conditions that mimic the syndrome in humans. Mercuric chloride was given to each of 16 rats to induce renal injury. This dose raised the serum creatinine concentration by 4-fold from 0.5 mg/dL at *time 0* to 2.0 mg/dL 16.5 hours later. Then one-half the animals were given the peptide (n=8) and the other half were given the vehicle (n=8), subcutaneously. The vehicle is sterile 0.01% borine serum albumin in phosphate-buffered saline, pH 7.40. On *day 2* of the syndrome, the creatinine concentration rose less in the peptide-treated rats (3.0 mg/dL) than in those receiving the vehicle (3.9 mg/dL) (P=0.0499). Importantly, on *day 3* the creatinine concentration declined to 2.6 mg/dL in animals given the peptide, but continued its climb to 4.2 mg/dL in rats given only the vehicle

(P=0.020). On *day 7*, survival appeared greater in animals treated with the peptide (88%) than the vehicle (63%).

Thus 14-Ser peptide speeds recovery of kidney function when administered **after** nephrotoxic renal injury is clinically detectable.

In a separate set of experiments, 14-Ser (n=7) or vehicle (n=9) was given to 16 rats 24 hours **before** mercuric chloride-induced injury. On day 3, renal function assessed using the serum creatinine concentration was significantly better in rats given the peptide (P=0.039). Survival on day 4 was 71% in rats treated with the peptide but only 22% in those given the vehicle (chi squared, P=0.049). Treatment with the 14-Ser peptide 24 hours before the mercuric chloride-induced insult appeared to have a more favorable effect on survival than when given 1 hour afterwards, although the difference did not quite achieve statistical significance.

To determine if the beneficial effects of 14-Ser peptide on survival and renal function were the result of the mitogenic action of the peptide, DNA synthesis was measured in the kidneys of rats given mercuric chloride. 5-Bromo-2'-deoxyuridine (BrdU), a nonradioactive analog of thymidine that is incorporated into DNA was used to label nuclei. A monoclonal antibody to BrdU and diamidobenzidene staining were used to detect nuclei undergoing DNA synthesis in histological sections of renal tissue. BrdU was dissolved in 20% ethanol and was injected intra-peritoneally 23 hours after administration of mercuric chloride. One hour later the kidneys were quickly perfused with a warm solution of phosphate-buffered saline via an aortic catheter to eliminate red blood cells from the organ and maintain patency of the renal tubules. The capsule was removed from the kidneys which were then bisected longitudinally, and fixed in formalin for 4 hours, and then in 70% ethanol. Tissue sections were prepared and BrdU-labeled nuclei were detected as described above.

BrdU staining of kidneys of rats given no mercuric chloride revealed about 3 labeled nuclei per high-power field (X400) in the subcapsular and aglomerular (inner) cortex. In the kidneys of a rats given mercuric chloride, extensive necrosis

of tubular cells of the terminal portion (S_3) of the proximal nephron was observed, as expected in this well-characterized model system. The extent of necrosis was somewhat variable from rat-to-rat but was largely confined to the aglomerular cortex. More labeled cells were seen in the aglomerular cortex in rats given mercuric chloride 24 hours earlier than in cells of untreated control rats. Renal tissue from a rat given 14-Ser (100 μg) 24 hours before administration of mercuric chloride was also examined. In this animal, 1 hour of BrdU labeling was carried out 24 hours after mercuric chloride as described herein. Microscopic examination revealed that many more labeled nuclei were present adjacent to the zone of tubular cell necrosis. This finding is important because it is in this boundary area of the cortex where regenerating cells are expected to undergo mitosis and then migrate into nephrons in the necrotic zone to replace cells that were irreversibly injured by the nephrotoxic insult. Thus the 14-Ser peptide appears to stimulate DNA synthesis in cells of regenerating nephrons after mercuric chloride-induced tubular necrosis, and by this mechanism, could thereby speed recovery of renal function and improve survival after ARF.

Example 3: 14-Ser Peptide is Effective in the Treatment of Ischemic Acute Renal Failure

Ischemic acute renal failure is often caused by low blood pressure in the setting of severe hemorrhage, fluid loss (diarrhea, vomiting, diuretics), infection (sepsis), and cardiac failure (myocardial disease, arrhythmia). It can also cause impaired function in the donor kidney after transplantation into its new host. To determine if 14-Ser peptide would serve as a therapeutic agent in animals with ischemic ARF, male rats weighing 200-225 gm at the start of the experiment were used. Animals were first given pentobarbital (12.5 mg/100 gm of rat) subcutaneously to induce anesthesia. The skin was then shaved, and small incisions were made over the right and then left flanks to expose the kidneys. A Schwartz surgical clamp was placed on each renal pedicle (ureter, artery, vein) to deprive the kidneys of blood flow to induce acute ischemic renal failure. About 45

minutes later the clamps were removed to permit reflow of blood into the injured kidneys, and metal skin clips were used to close the skin incisions. This is an established model of experimental ARF, as described herein. One hour after removing the two clamps, each rat received either 14-Ser peptide (AQPYPQGNHEASYG) (100 μg) in vehicle (0.01% bovine serum albumin in phosphate-buffered saline) or vehicle alone, injected subcutaneously at the nape of the neck.

To ascertain the effect of the peptide on survival and recovery of kidney function, bilateral renal artery clamping was used to induce ischemic ARF in 29 rats. WGF-derived peptide (14-Ser, 100μg) was administered s.c. to each of 14 animals 1 hour after the 45-minute ischemic insult was terminated; 15 rats received an equal volume of vehicle. Survival appeared greater in rats given the peptide (93%) than in animals receiving the vehicle (67%) (chi squared, P=0.082) on *day 6* after induction of ischemic ARF (FIG. 3A). Blood was obtained from the tail vein on the days indicated on the figure and the serum creatinine concentration was measured. Accelerated recovery of kidney function in peptide-treated rats was manifested by the lower serum creatinine concentration on *days 1, 2, 3 and 4* of the ARF syndrome. By *day 1* the creatinine concentration had increased from its basal value of 0.5 mg/dL to 3.8 mg/dL in rats given the vehicle (n=15 rats), but reached a significantly lower value of 3.4 mg/dL in animals treated with peptide (n=14 rats) (P=0.032, Student's *t*-test) (FIG. 3B). The creatinine concentration was lower in peptide-treated animals than those receiving the vehicle on *day 2* (P=0.015), and *day 3* (P=0.014). By *day 4*, the creatinine concentration had fallen progressively to a value of 2.2 mg/L in peptide-treated rats, whereas it increased to a 2-fold higher value of 4.4 mg/dL in animals given the vehicle (P=0.011). Thus, during the first four days of the ARF syndrome, kidney function remained severely impaired in animals given the vehicle, whereas peptide-treated rats made a rapid recovery.

In summary, 14-Ser WGF-derived peptide administered to rats with either ischemic or nephrotoxic kidney injury improves survival and speeds recovery from, the acute renal failure syndrome.

Example 4: Improved Survival was Achieved with 11-mer WGF-Derived Peptide Treatment of Nephrotoxic Acute Renal Failure

Experimental ARF was induced in rats by subcutaneous injection of mercuric chloride. The synthetic peptide AQPYPEGNHEA (dissolved in sterile 0.01% bovine serum albumin and PBS) was injected subcutaneously into rats two hours after mercuric chloride was given to demonstrate its beneficial effect on survival from the ARF syndrome. In one experiment a total of 17 rats were injected with the dose of mercuric chloride shown to induce ARF as described above. Then different amounts of the peptide (known to stimulate growth of kidney cells in culture) were injected into the animals 1 hour later to assess its capacity to improve the outcome. Three days later, five of five animals given saline alone and three of three rats given 50 micrograms of the peptide were dead. In contrast, two of three rats given 100 micrograms of peptide were alive, as were five of six given 200 micrograms. In summary, none of eight rats (0%) given up to 50 micrograms of peptide survived, whereas seven of nine (78%) given 100-200 micrograms did (chi squared, $P = 0.032$). Thus, the 11-mer WGF peptide improves survival in nephrotoxic ARF.

Example 5: Effect of Specific WGF-Derived Peptides on Kidney Repair

In addition, administration of specific WGF-derived peptides speeds repair of renal structure. Standard assays in renal growth physiology and pathology are used to demonstrate that the growth-promoting (mitogenic) effect of a WGF peptide stimulates an increased number of kidney cells to initiate synthesis of DNA in preparation for cell division, as has been shown in tissue culture.

Initially, sections of kidney tissue from rats with mercuric chloride-induced ARF are prepared and inspected under light microscopy to compare the extent of renal injury and repair in animals that received WGF peptide and those that received a saline vehicle alone. Another measure of the effect of WGF on recovery is an histopathological assessment based on detailed microscopic inspection of kidney tissue. A scoring system that grades the characteristic features of ARF and recovery is used to assess and compare kidney tissue from rats that did or did not receive treatment with WGF (Miller *et al.*, 1994).

Depending upon methodologic considerations, radioactive thymidine can be used to measure the capacity of WGF peptide to stimulate renal DNA synthesis after ARF. In these experiments radioactive thymidine is injected intraperitoneally 1 hour before death, and its incorporation into renal DNA is determined by subsequently extracting DNA from the tissue, and then measuring the amount of DNA by a chemical assay, and its radioactivity by scintillation counting. Autoradiograms can also be prepared and used to determine which cells in kidney tissue have incorporated radioactive thymidine into DNA. All the techniques referred to are known to those of skill in the art (Coimbra *et al.*, 1990).

Example 6: Inhibition of WGF-Derived Peptide Mitogenesis by TGF-β2 and YPQGN Peptide

To better understand the role of the 14-Ser peptide in renal cell growth regulation, agents that could inhibit its mitogenic effect were studied.

Transforming growth factor (TGF)-β2 is an autocrine growth inhibitor secreted by BSC-1 cells that limits proliferation in culture. Exogenous TGF-β2 was added to the culture medium to determine its capacity to inhibit the growth-promoting effect of 14-Ser. TGF-β2 at a concentration of 1 ng/ml was sufficient to abolish the 25% stimulation of growth induced by 14-Ser. At a TGF-β2 concentration of 2 ng/ml, growth was inhibited by 30% in the presence or absence of 14-Ser; at 10 ng/ml, inhibition was 60%. At a TGF-β2 concentration of 6 ng/ml, exogenous 14-Ser up to 5 μg/ml did not reverse growth inhibition.

Because TGF-β2 inhibits proliferation induced by diverse mitogens, a more specific inhibitor was sought. Previously, studies of the 5-amino acid WGF-derived peptides YPQGN and PQGNH were shown to inhibit the mitogenic effect of the 6-amino acid peptide, YPQGNH. These pentapeptides do not alter growth when added to cells. Cells were preincubated for 30 minutes with the pentapeptide YPQGN. The culture medium was then aspirated, the monolayer was rinsed, fresh medium was added, and the number of cells counted 4 days later. Preincubation of cells with YPQGN inhibited the mitogenic effect of the 6-amino acid peptide. In addition, preincubation with YPQGN for 10 or 30 minutes completely blocked the growth-promoting effect of each of the following peptides: CPQGNH, 14-Ser, 28-mer (14-Ser dimer), and the 40-mer (Table 3). Importantly, the mitogenic effect of partially-purified Wound Growth Factor (passed over an heparin-affinity cartridge, not an HPLC column) was also completely abolished.

These observations suggest that native WGF (partially-purified) and WGF-derived peptides 6 to 40 amino acids in length containing the sequence Y/CPQGNH described herein, bind to the same cell surface receptor, which also has high affinity for the pentapeptide YPQGN.

Additional experiments were carried out to determine if the capacity of YPQGN to block the growth-promoting effect of native WGF or 14-Ser peptide was specific. Cultures of BSC-1 cells were preincubated for 30 minutes with YPQGN, the medium was then aspirated, and the monolayer was rinsed twice with fresh medium. Each of the following renal cell mitogens at concentrations known to be maximal under this set of conditions was assessed individually: EGF (50 ng/ml), insulin-like growth factor-I (50 ng/ml), vasopressin (75 pg/ml), acidic fibroblast growth factor (100 pg/ml), and basic fibroblast growth factor (1,000 pg/ml). The results indicated that each of the five proteins was fully mitogenic despite pre-exposure of the cells to YPQGN. Pretreatment with YPQGN also failed to block the growth-inhibitory effect TGF-β2 (6 mg/ml). These observations indicate that mitogenic inhibition of native WGF by YPQGN is specific, and that

the WGF receptor site and signal transduction pathway differs from that of the five growth factors tested.

Cloning the cell surface receptor for WGF protein and WGF-derived peptides.

The information summarized above suggests an experimental strategy to isolate and clone the cell surface receptor to which native WGF and WGF-derived peptides bind. To detect the WGF receptor on the surface of monkey kidney epithelial cells of the BSC-1 line, the high affinity of the 40-mer WGF peptide is exploited which in the presence of heparin binds to the cells with a $K_{1/2}$ = 0.0051 μM (5.1 nM). In addition, the specificity of binding to cells or plasma membrane fractions is monitored by using the pentapeptide YPQGN which blocks adherence of the peptide to the cell surface. Radioiodinated 40-mer is prepared and then added with heparin to cultures of BSC-1 cells. The receptor is affinity labeled by cross-linking it to [^{125}I]40-mer by the action of disuccinimidyl suberate (Segarini *et al.*, 1987). The cell proteins are solubilized and separated by SDS-PAGE. Autoradiography enables visualization of the receptor and definition of its size. After a sufficient amount of receptor protein is isolated by this gel purification method, amino-terminal and internal amino acid sequence information will be obtained, oligonucleotide primers prepared, and the polymerase chain reaction used to obtain a cDNA clone encoding the receptor protein.

Example 7: *In Vivo* Comparison Between the 14-Ser WGF-Derived Peptide and EGF on Survival After Acute Renal Failure

The efficacy of the 14-Ser peptide as a novel therapeutic agent in mercuric chloride-induced ARF was compared to EGF, which was the first peptide growth factor shown to be effective in this animal model system (Coimbra *et al.*, 1990).

Eight rats in each of three groups (n=24) were given either 14-Ser (100 μg, s.c.) 1 hour after mercuric chloride (2.25 mg/kg s.c.), EGF (20 μg, s.c.) 2 hours after the toxin, or the vehicle (s.c.), and survival was monitored. Four days later,

63% of rats given the 14-Ser peptide were alive compared to 25% of animals receiving the vehicle (chi squared, P=0.0499); 50% of rats receiving EGF were alive (chi squared, P=N.S.). On *day 5*, 50% of rats given EGF were alive, compared to 12.5% of animals given the vehicle (chi squared, P=0.0436). On *day 6*, survival was identical in rats given the 14-Ser peptide or EGF (37.5%) compared to 12.% of animals treated with the vehicle. These observations indicate that the 14-Ser peptide and EGF given after renal injury are similar in their ability to improve survival from the ARF syndrome.

In a separate experiment, rats were given EGF (20 μg, s.c.) 24 hours prior to administration of mercuric chloride to compare the effect of this growth factor to the 14-Ser peptide. Surprisingly, EGF treatment was not different than the vehicle, whereas the 14-Ser peptide is highly protective under these conditions. Thus, on *day 4*, survival of rats given EGF was 25%, for vehicle, 31%, and for the 14-Ser WGF-derived peptide, 73%.

These studies indicate that the 14-Ser peptide is more effective than EGF in promoting survival when administered prophylactically, *i.e.*, before the onset of renal injury. Administration of WGF-derived peptide could prove particularly efficacious: (1) prior to surgical procedures in patients with a high risk of developing ARF (*e.g.*, the elderly, neonates), (2) in patients given potentially nephrotoxic agents such as antibiotics for treatment of sepsis or antineoplastic agents, and (3) for perfusion of donor kidneys prior to their transplantation into new hosts.

A WGF-derived peptide such as 14-Ser or native WGF dissolved in an aqueous solution can be used to perfuse and protect and isolated ("ex vivo") ischemic, "donor" kidney from injury which may occur upon reperfusion of blood following reimplantation into a human recipient. Thus WGF can serve as an effective protectant when added to solutions employed to preserve cadaveric kidneys or kidneys removed from living donors and intended for transplantation.

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WE CLAIM:

1. A protein having the following characteristics:

a) an estimated molecular weight of about 45 kDa, said estimate obtained by electrophoresing the HPLC-purified protein on an SDS-polyacrylamide gel;

b) capability of stimulating mitogenic activity when in contact with cultured cells; and

c) released by BSC-1 cells in culture by scrape-wounding.

2. The protein of claim 1 having a partial amino acid sequence at the amino terminal end of NH_2-alanine-glutamine-proline-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-glutamic acid-X-alanine/ serine-tyrosine-glycine-COOH.

3. A protein having the following characteristics:

a) an estimated molecular weight of about 22 kDa, said estimate obtained by electrophoresing the HPLC-purified protein on an SDS-polyacrylamide gel;

b) capability of stimulating mitogenic activity when in contact with cultured cells; and

c) released by BSC-1 cells in culture by scrape-wounding.

4. The protein of claim 3 having a partial amino acid sequence at the amino terminal end of NH_2- alanine-glutamine-proline-tyrosine/cysteine-proline-glutamine-glycine-asparagine-histidine-glutamic acid-alanine-threonine-serine-serine-serine-phenylalanine-COOH.

5. A peptide comprising an amino acid sequence of NH_2-tyrosine/ cysteine-proline-glutamine-glycine-asparagine-histidine-COOH.

6. The peptide of claim 5 having a length of from 7 to 16 amino acids.

7. A peptide having an amino acid sequence selected from the group consisting of:

AQPY/CPQGNHEATSSSF;
AQPY/CPQGNHEATSSS;
AQPY/CPQGNHEA;
AQPY/CPQGNHEAT;
AQPY/CPQGNHEATS;
AQPY/CPQGNHEATSS;
AQPY/CPQGNHEAAYG;
AQPY/CPQGNHEAAY;
AQPY/CPQGNHEAA;
AQPY/CPQGNHEA
AQPY/CPQGNHE;
AQPY/CPQGNHEASYG;
AQPY/CPQGNHEASY;
AQPY/CPQGNHEAS;
AQPY/CPQGNH;
QPY/CPQGNHE;
PY/CPQGNHEA;
QPY/CPQGNH;
PY/CPQGNHE;
Y/CPQGNHEA;
PY/CPQGNH
Y/CPQGNHE;
Y/CPQGNHEATSSSF;
Y/CPQGNHEATSSS;
Y/CPQGNHEATSS;

Y/CPQGNHEATS and
Y/CPQGNHEAT.

8. A composition comprising the protein of claim 1.

9. A composition comprising the protein of claim 1.

10. A composition comprising the peptide of claim 7.

11. A protein comprising the peptide YPQGNH or CPQGNH.

12. A protein comprising the peptide AQPYPQGNHEASYG or AQPCPQGNHEASYG.

13. A method for producing a protein having the following characteristics:

i) an estimated molecular weight of about 45 kDa, said estimate obtained by electrophoresing the HPLC-purified polypeptide on an SDS-polyacrylamide gel;

ii) capability of stimulating mitogenic activity when in contact with cultured cells; and

iii) released by BSC-1 cells in culture by scrape-wounding;

said method comprising:

a) culturing kidney epithelial cells in media,

b) scape-wounding the cells in culture, and

c) obtaining the protein from the conditioned media.

14. The method of claim 11, wherein the protein obtained is isolated from the conditioned media and purified.

15. The method of claim 11, wherein the kidney epithelial cells in culture are from the BSC-1 African green monkey kidney epithelial cell line.

16. A method for producing a protein having the following characteristics:

i) an estimated molecular weight of about 22 kDa, said estimate obtained by electrophoresing the HPLC-purified polypeptide on an SDS-polyacrylamide gel;

ii) capability of stimulating mitogenic activity when in contact with cultured cells; and

iii) released by BSC-1 cells in culture by scrape-wounding;

said method comprising:

a) obtaining kidney epithelial cells in culture,

b) scrape-wounding the cells in culture, and

c) allowing medium in which the scrape-wounded cells are cultured to become conditioned.

17. An antibody to the protein of claim 1.

18. An antibody to the peptide AQPYPQGNHEASYG or AQPCPQGNHEASYG.

19. An antibody to a peptide of claim 7.

20. The antibody of claim 17, wherein the antibody is a monoclonal antibody.

21. The antibody of claim 19, wherein the antibody is a monoclonal antibody.

22. A method of making the protein of claim 1, said method comprising:

a) obtaining a nucleotide sequence encoding the protein or peptide; and

b) using the nucleotide sequence in a genetic expression system to make the protein or peptide.

23. A diagnostic kit to measure the quantity of a WGF protein or a mitogenic peptide therefrom, in a biological sample, said kit comprising in separate containers:

a) an antibody to WGF or to a mitogenic peptide therefrom; and

b) a means for detecting a specific complex between the WGF protein or a mitogenic peptide and the antibody.

24. The method of claim 23, wherein the mitogenic peptide has the sequence tyrosine/cysteine-proline-glutamine-glycine-asparagine histidine.

25. A method of treating a person with acute renal failure, said method comprising a) preparing a pharmacologically effective amount of native WGF protein or WGF-derived peptide in a suitable diluent; and b) administering the preparation to the person.

26. The method of claim 25, wherein WGF is ligated to a cytolytic ligand for treatment of kidney cancer.

27. The method of claim 25, wherein the cytolytic ligand comprises a toxin for treatment of kidney cancer.

28. A method of perfusing "ex vivo" a donor human kidney, intended for transplantation into a new host, with a pharmacologically effective amount of native WGF protein or WGF-derived peptide in a suitable diluent.

29. A method of treating a person with chronic renal disease, said method comprising a) preparing a pharmacologically effective amount of native

WGF protein or WGF-derived peptide in a suitable diluent; and b) administering an effective amount of the preparation to the person.

30. A composition comprising at least one multimer of a WGF-derived peptide, said peptide selected from the group consisting of the hexapeptide NH_2-Y/CPQGNH-*COOH*, the first 10 amino acids of the 22-kD and 45-kD WGF protein (AQPYPQGNHE), the 14-Ser peptide (AQPYPQGNHEASYG) and other WGF sequences, and combinations thereof.

31. A composition comprising at least one WGF-derived peptide in which at least one amino acid is replaced with a naturally-occurring amino acid, an amino acid derivative, or a non-native amino acid so that the resulting peptide retains its mitogenic activity.

32. A composition comprising at least one WGF-derived peptide, said peptide having an amino acid sequence that differs by at least one amino acid substitution, deletion, or insertion from a WGF-derived peptide having mitogenic activity.

33. A composition comprising a peptide or protein that is structural homolog of a WGF-derived peptide or protein, said peptide or protein having a nucleotide and amino acid sequence with at least 80% homology to the WGF-derived peptide or protein, said composition exhibiting mitogenic activity.

34. A composition comprising at least one structurally altered isoform of a WGF-derived peptide or protein, said isoform posttranslationally modified by one of the following mechanisms, glycosylation, sulfation or myristilation, said composition having mitogenic activity.

35. A composition comprising a cell surface receptor(s) to which native WGF protein and WGF-derived peptides bind to initiate mitogenic signal transduction.

36. A composition comprising a WGF protein or at least one WGF-derived peptide in combination with other known growth factors and/or nutrients.

37. The composition of claim 36 used in treating a person with acute renal failure or chronic renal disease.

38. A pentapeptide that blocks the mitogenic effect of native WGF protein and WGF-derived peptides.

39. The pentapeptide of claim 38, having an amino acid sequence of YPQGN.

39. The pentapeptide of claim 38, having an amino acid sequence of PQGNH

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/10574

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/475 A61K38/18 A01N1/02 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 476 922 A (TOBACK F GARY ET AL) 19 December 1995	1,2,8,9, 13,14, 17,20, 22,23,33
Y	whole document, esp. claim 1, Fig. 5 (43kDa), column 12, line 12, column 15	3-7, 10-12, 15,16, 18,19, 21,24
Y	US 5 135 856 A (TOBACK F GARY ET AL) 4 August 1992	3-7, 10-12, 15,16, 18,19, 21,24
	column 6/7, claim 1	

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 December 1998	30/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kronester-Frei, A

2

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Int onal Application No
PCT/US 98/10574

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DIJKE TEN P ET AL: "GROWTH FACTORS FOR WOUND HEALING" BIO/TECHNOLOGY, vol. 7, no. 8, 1 August 1989, pages 793-798, XP000073312 whole document	1-39
A	HAMMERMANN M.R. ET AL.: "Role of Growth factors in regulation of renal growth" ANU.REV.PHYSIOL., vol. 55, 1993, page 305-21 XP002085940 whole document	1-39
A	WANG, S. ET AL: "Role of growth factors in acute renal failure" NEPHROL., DIAL., TRANSPLANT. (1997), 12(8), 1560-1563 CODEN: NDTREA;ISSN: 0931-0509, XP002085941 whole document, esp. Fig. 1	1-39
A	US 5 618 917 A (TOBACK F GARY ET AL) 8 April 1997 claim 1	1-24,28
A	WO 93 07900 A (CANTAB PHARMA RES) 29 April 1993 CLAIMS	28
A	PATENT ABSTRACTS OF JAPAN vol. 1, no. 1 & JP 03 128330 A (TAKEDA CHEM IND LTD), 31 May 1991 WHOLE ABSTRACT see abstract	28
A	-& JP 03 128330 A (TAKEDA CHEM IND LTD) 31 May 1991 SEQUENCES	28

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ɔnal Application No
PCT/US 98/10574

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